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The effect of genetic variation on asthma severity and treatment in childhood

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The effect of genetic variation on asthma severity and treatment in childhood

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University of Dundee

THE EFFECT OF GENETIC VARIATION ON ASTHMA SEVERITY AND TREATMENT IN CHILDHOOD

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CONTENTS

LIST OF ILLUSTRATIONS 5

LIST OF TABLES 7

LIST OF ABBREVIATIONS 10

ACKNOWLEDGEMENTS 12

SECTION I: INTRODUCTION

Hypothesis 14

Objectives 15

Summary 16

SECTION II: BACKGROUND

Chapter 1: Asthma severity and burden of the disease in childhood 18

Chapter 2: The BREATHE study 21

Chapter 3: Pharmacological management of childhood asthma 23

Chapter 4: The underlying genetics of asthma 28

Chapter 5: β_2 adrenergic receptor gene variations and asthma 30

Chapter 6: Filaggrin null mutations in childhood asthma 33

Chapter 7: Epithelial permeability and filaggrin 36

SECTION III: METHODS

Chapter1: Personal interview 39

Chapter 2: Pulmonary function tests 44

Chapter 3: Saliva collection for DNA and genotyping 46

Chapter 4: Measurement of transepidermal water loss 54

Chapter 5: Dual sugar test 57

Chapter 6: Statistical Analyses 58

SECTION IV: RESULTS

Chapter 1: Description of the BREATHE cohort 62

Chapter 2: Effects of β_2 adrenergic receptor gene variations in asthma exacerbations 70

Chapter 3: Effects of filaggrin null mutations in asthma exacerbations 90

Chapter 4: Effects of filaggrin defects on epithelial permeability 102

SECTION V: DISCUSSION

Chapter 1: BREATHE is a large cross-sectional study in children's asthma with genotype and phenotype data 107

Chapter 2: Adrenergic β_2-receptor genotype predisposes to exacerbations in steroid-treated asthmatics on frequent salbutamol or salmeterol 110

Chapter 3: Filaggrin null mutations are associated with increased asthma exacerbations in children and young adults 118

Chapter 4: Epithelial permeability is increased in children with filaggrin-related barrier defects in skin but not gut 121

Chapter 5: Future work 124

SECTION VII: CONCLUSION 131

SECTION VIII: REFERENCES 140

APPENDIX 163

LIST OF ILLUSTRATIONS

Figure	Title of the figure	Page
3.1.1	The BREATHE study questionnaire-page1	42
3.1.2	The BREATHE study questionnaire-page2	43
3.3.1	Oragene® DNA collection kit	47
3.3.2	Method of collection of saliva in the Oragene® DNA collection kit in children older than 5 years of age	48
3.3.3	Collection of saliva by Oragene® DNA collection kit in a child under 5 years of age	49
3.3.4	Method of cutting the saliva sponge and placing in the kit	50
3.4.1	Tewameter and the probe	56
3.4.2	Graphical representation of the transepidermal water loss measurement	56
4.1.1	Pie chart illustrating the exposure to animals	67
4.1.2	Medications prescribed for asthma control in Tayside and Dumfries	68
4.1.3	Common allergens positive on skin prick test	69
4.2.1	Association of airway obstruction (FEV_1/FVC) and β_2 adrenergic receptor gene variations in children with mild (treatment steps 0 and 1) and moderate to severe (treatment steps 2,3,4) asthma	89
4.3.1	The proportion of participants with *FLG* null allele frequency across the treatment steps of management of asthma	95
4.3.2	Proportion of individuals with *FLG* null mutations with and without asthma exacerbations over the previous 6 months	95

Figure	Title of the figure	Page
4.3.3	The proportion of participants with exacerbations across the steps of management of asthma	100
4.3.4	Comparison of asthma exacerbations between children with and without *FLG* null mutation across the treatment steps of asthma	101
4.4.1	Transepidermal water loss in the children heterozygous for the *FLG* null allele with asthma and the control population	105
4.4.2	Excreted lactulose/ mannitol ratio in the children heterozygous for the *FLG* null allele with asthma and the control population	106

LIST OF TABLES

Table	Title of the table	Page
4.1.1	Demographic and clinical characteristics of participants for the BREATHE study	64
4.1.2	Family history of asthma, eczema and allergic rhinitis in the study population	65
4.1.3	Personal history of the participants of the BREATHE study	66
4.2.1	Demographics of participants for the association study between the β_2 agonist genotype variations and asthma exacerbations	74
4.2.2	Medication use and exacerbations of asthma	75
4.2.3	Genotype distributions for codons 16 and 27	76
4.2.4	Overall effect of Arg16Gly genotype on oral steroid intake due to asthma exacerbations, in children and young adults with asthma regardless of treatment	77
4.2.5	Overall effect of Arg16Gly genotype on school absences due to asthma exacerbations in children and young adults with asthma regardless of treatment	78
4.2.6	Overall effect of Arg16Gly genotype on hospital admissions due to asthma exacerbations in children and young adults with asthma regardless of treatment	79
4.2.7	Overall effect of Arg16Gly genotype on overall asthma exacerbations, in children and young adults with asthma regardless of treatment	80
4.2.8	Overall effect of Glu27Gln genotype on oral steroid intake in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype	81

Table	Title of the table	Page
4.2.9	Overall effect of Glu27Gln genotype on school absences due to asthma exacerbations in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype	82
4.2.10	Overall effect of Glu27Gln genotype on hospital admissions due to asthma exacerbations in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype	83
4.2.11	Overall effect of Glu27Gln genotype on overall asthma exacerbations in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype	84
4.2.12	Association of asthma exacerbations and Arg16Gly genotype variations according to the steps of management of asthma	85
4.2.13	Association of asthma exacerbations and Glu27Gln genotype variations according to the steps of management of asthma conditioned on Arg16Gly genotype	86
4.2.14	Effect of Arg16Gly genotype status on the proportion of patients developing asthma exacerbations according to salmeterol treatment status.	87
4.2.15	Association and drug-genotype interaction of Arg16Gly genotype with asthma exacerbations in children with, and without, daily exposure to β_2 agonists.	88
4.3.1	Demographic characteristics of participants with asthma for the study of association between asthma exacerbations and filaggrin mutations	93
4.3.2	Medication use, genotypes and asthma exacerbations	94
4.3.3	Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus exacerbations of asthma.	96

Table	Title of the table	Page
4.3.4	Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus oral short courses of oral steroids for treatment of asthma exacerbations in the previous 6months	97
4.3.5	Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus asthma-related school absences in the previous 6 months	98
4.3.6	Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus asthma-related hospital admissions in the previous 6months	99
4.4.1	Characteristics of controls and participants with filaggrin mutations for permeability study	104

LIST OF ABBREVIATIONS

ADRB2: Adrenergic $\beta_2$receptor agonist gene

AMP: Adenosine monophosphate

BDP: Beclomethasone dipropionate

BMI: Body Mass Index

BTS: British Thoracic Society

CI: Confidence interval

Cys LT: Cysteinyl leukotriene

FDA: Food and Drug Administration

FEV_1: Forced expiratory volume in 1 second

FEF_{25-75}: Forced expiratory flow 25%-75%

FVC: Forced vital capacity

FLG: Filaggrin encoding gene

GM CSF: Granulocyte macrophage colony stimulating factor

GWA: Genome wide association

HER2: Human epidermal growth factor receptor2 encoding gene

KDa: Kilo Dalton

MAD: Median of the absolute deviation

MAF: Minor allele frequency

MMP12: Matrix metalloproteinase 12

NHS: National Health Service

OR: Odds ratio

PAQLQ: Paediatric Asthma Quality of Life Questionnaire

PCR: Polymerase chain reaction

PEFR: Peak expiratory flow rate

PPARγ: Peroxisome proliferator-activated receptor γ

qid: Quater in die (4 times daily)

SD: Standard deviation

SEM: Standard error of mean

SNP: Single nucleotide protein

SMART: Salmeterol Multicenter Asthma Research Trial

TEWL: Transepidermal water loss

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SECTION I

INTRODUCTION

Hypotheses

The thesis aims to explore the role of gene variation on asthma control in children and young adults. In order to achieve this, I have focused on the role of two common gene mutations that could have significant functional roles in this context. I have performed further studies to explore the biological role of one of the two mutations. The thesis explores the following three key hypotheses:

1. There is an association between the presence of the Arg16 variation on the β_2 adrenergic receptor gene (*ADRB2*) and the daily use of any inhaled β_2-agonist (as required salbutamol and/or regular salmeterol) on the risk of asthma exacerbations.
2. There is increased risk of asthma exacerbations in the children and young adults with filaggrin null mutations.
3. The barrier function of epithelia in the skin and gastrointestinal tract is reduced in asthmatic children with filaggrin mutations.

Objectives

1. To create a database containing information on genotype and clinical phenotype in children with asthma

2. To test the overall effect of the Arg16 allele on the β_2 adrenergic receptor gene (*ADRB2*) on the individual measures of asthma exacerbations and on the overall risk of exacerbations.

3. To test the effect of the genotype variations of *ADRB2* on the daily use of any β_2-agonists

4. To study the association between R501X and 2282del4 alleles of filaggrin null mutations and the risk of asthma exacerbations

5. To explore the distribution and function of filaggrin protein in the human skin and intestine, in order to help identify the primary site of allergen entry in humans.

Summary

1. I have described a population of children and young adults with asthma in primary and secondary care, in terms of relevant history, medication use and exacerbations.

2. My thesis presents observations reported for the first time that asthmatic children and young adults homozygous for the Arg16 allele on the β_2 adrenergic receptor gene (*ADRB2*), on frequent doses of on demand short-acting β_2-agonists are at greater risk of asthma exacerbations.

3. I have shown an increase in the risk of exacerbations per copy of Arg16 allele in children and young adults with asthma on the regular long-acting β_2-agonist salmeterol.

4. I have shown that there is an increase in risk of exacerbations per copy of Arg16 allele in children and young adults with asthma on frequent (once daily or more) as required doses of inhaled salbutamol. This effect is not observed on participants with asthma who are not exposed to β_2-agonist on a daily basis.

5. I have shown that the Arg16Arg variant status may be associated with worse airway obstruction, as measured by the FEV_1/FVC ratio.

6. I have shown that the individuals with *FLG* null alleles have a significantly increased risk of exacerbations requiring hospital admissions, courses of oral steroids, or experiencing school absences.

7. The permeability of the skin, as measured by transepidermal water loss, is around 50% higher in children heterozygous for filaggrin gene defects compared to clinically unaffected children but the permeability of the intestinal barrier, as measured by dual sugar absorption tests, is not different. My observations provide for the first time, an explanation of the clinical basis for the possible increased risk of allergen entry in children with eczema and asthma.

SECTION II

BACKGROUND

Chapter 1

Asthma Severity And Burden Of The Disease In Childhood

Asthma is one of the most common chronic diseases in the world[1,2,3,4]. Genetic and environmental factors contribute to the development of asthma and its subsequent severity. Asthma is a complex syndrome with variable outcomes and this variability has made it difficult to appropriately monitor and control the condition. Multifaceted markers are necessary to adequately characterise this complex disorder[5]. There is also need to identify primary prevention strategies to control asthma exacerbations through interventions that promote adherence to asthma guidelines and reduce exposures to causes of asthma exacerbations[6].

Over 5million people in the UK have asthma; 1.4 million of them are children under 16 years of age[7]. Scotland tops the ranking list by country for the prevalence of current asthma symptoms in the childhood and teenage years[8]. In England, an estimated 261,400 people were newly diagnosed with asthma in 2005; 5.7 million people had an asthma diagnosis and were prescribed 32.6 million asthma-related prescriptions[9]. A better understanding of the variability in disease and response to treatment, and interactions between the pathology

of disease and its clinical expression is essential to the appropriate selection of therapy and the maximisation of outcomes in children and young adults.

Exacerbations of asthma are the commonest cause of medical admissions in childhood and have significant effects on quality of life[10]. Exacerbations represent periods of enhanced airway inflammation and remodelling[11] and are a marker of pulmonary decline[12]. Potentially life-threatening asthma exacerbations are of great concern to individuals with asthma. They diminish the quality of life of the patients and their families[10, 13, 14]. 2.1 million adults and 500,000 children in the UK have severe asthma symptoms including debilitating shortness of breath, attacks so bad that they are unable to speak, fear of death and emergency hospital admissions[15]. There were 40 deaths in children from asthma exacerbations in 2004 in the UK[16]. In children with asthma, school absences from asthma[17], use of short courses of oral steroids[18, 19] and asthma-related hospital admissions[20] represent well-validated measures of asthma exacerbations. I have combined these measures to develop a tool for defining asthma exacerbations that has now been validated through previously published work[21-26].

Commonly used non-invasive measures to detect clinical deterioration include peak expiratory flow rate (PEFR), asthma symptom scores and increase in daily requirement of salbutamol as reliever medication[23]. My research underlines the importance of genotyping for common genotypes in children with asthma, to improve quality of life and prevent asthma exacerbations. An understanding of the possible relationship between gene defects, inheritance and asthma might generate two newer hypotheses that primary prevention

strategies for asthma may be more cost effective and beneficial for genotype-stratified population in comparison to current treatments directed at unstratified populations[27].

Chapter 2

The BREATHE Study

Paediatric pharmacogenetic studies have the potential of improving the quality of medical care for children[28]. Genetic variations, such as in the genes encoding for the β_2 adrenergic receptor (*ADRB2*), the gene encoding for the filaggrin protein (*FLG*), gene encoding for the matrix metalloproteinase 12 (*MMP12*), chitinase 3-like1 gene (*CHI3L1* rs495028) have been shown to influence asthma phenotype, possibly through an interaction with pharmacological treatments[11, 21, 23, 29, 30]. Common functional polymorphic variants of the β_2 adrenergic receptor gene are known to affect the response to asthma medication[31].

Previous studies have shown that prescribing asthma medications in primary and secondary care in the UK are not always consistent with guideline recommendations[32, 33]. Off-label prescribing for children with asthma in UK primary care is associated with worse asthma control[34]. In addition, the genotypic variations that can affect medication use are not considered in these guidelines.

A database of children and young adults with physician diagnosed asthma was developed in Tayside and Dumfries. The database contains information on patient genotype, environmental factors contributing to asthma, and clinical asthma severity as defined by asthma medication use and frequency of asthma exacerbations[21-25]. The aim was to study the association between genotypic variation and clinical asthma phenotype to create a large

population-based database of this information. It was also anticipated that the database will be studied aiming to explore therapeutic response to asthma medication; and relating clinical effect (including drug response) to challenge test and genotyping results in the longer term. I continued recruitment to the BREATHE study during my period of research. Now the study has also been extended to Sussex to explore the variations in the clinical phenotype and genotype characteristics of asthma in a wider population across the UK.

Chapter 3

Pharmacological Management Of Childhood Asthma

The principles underlying therapy of asthma have remained unchanged over the past several decades. The aims of pharmacological management are to control symptoms including nocturnal symptoms and exercise-induced asthma; prevent exacerbations; achieve best possible pulmonary function; and minimise side effects. Bronchodilator drugs like short-acting β_2 adrenergic receptor agonists are used as reliever to reverse the bronchospasm of an asthma attack. Anti-inflammatory drugs like gluco-cortocoids are used to control bronchial inflammation in an effort to reduce the severity and frequency of asthma attacks. Patients with acute exacerbations of asthma are often managed by short courses of oral steroids. In patients who remain symptomatic despite inhaled steroid therapy, long-acting β_2 adrenergic receptor agonists may be added as controller to the steroid regimen with good success. Agents like leukotriene-receptor antagonists, are directed at specific mechanisms underlying the initiation or progression of asthma and are also used as controller medication for asthma.

- **The British Thoracic Society (BTS) guidelines for asthma management**

Asthma in children is managed in the stepwise approach according to the British Thoracic Society (BTS) guidelines[18, 35] in the UK. Initial pharmacologic treatment of childhood

asthma consists of 'reliever' medication (short-acting β_2-agonists) according to need (step 1, BTS guidelines) [18]. The regular 'controller' therapy starts with the daily use of inhaled steroids (BTS step 2). When asthma control on BTS step 2 is inadequate, inhaled long-acting β_2 -agonists, e.g. salmeterol is added (BTS step 3)[18]. However, if control is still inadequate and there is no response to long-acting β_2 –agonists, leukotriene receptor antagonists, e.g. montelukast or slow-release theophylline are added (BTS step 3)[18]. For persistent poor control, the steroid dose is increased to 800μg/day (BTS step 4)[18]. Finally for children requiring frequent short courses of oral steroids despite highest dose of inhaled steroids and add-on therapy, regular oral steroids are commenced (BTS step 5)[18]. Common measures of asthma control include the occurrence of day-to-day asthma symptoms, 'breakthrough' asthma attacks, the need for 'reliever' treatment with short-acting β_2 agonists, and quality-of-life[18].

For the purpose of my studies, the asthma prescribing level was modified for physician-led management of asthma, as follows: step 0 – no use of inhaled salbutamol on demand within the past month; step 1 - inhaled short-acting β_2 -agonists eg. salbutamol on demand; step 2 - regular inhaled steroids plus inhaled salbutamol on demand; step 3 - regular inhaled long-acting β_2 -agonists eg. salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand.

- **β_2 adrenergic receptor agonists**

The drugs that are most effective in relaxing the airway smooth muscle and reversing bronchoconstriction are the β_2 adrenergic receptor agonists. They are the preferred treatment for rapid symptomatic shortness of breath associated with bronchoconstriction in patients with asthma[36, 37]. Long acting β_2 agonists like salmeterol, act by directly relaxing airway smooth muscles and consequently causing bronchodilation. Although human bronchial smooth muscle receives little or no sympathetic innervation, it nevertheless contains large numbers of β_2 adrenergic receptors. Stimulation of these receptors activates the G_s adenylyl cyclase- cyclic AMP pathway with a consequent reduction in smooth muscle tone[38]. β_2 adrenergic receptor agonists also increase the conductance of large Ca^{2+} sensitive K^+ channels in airway smooth muscles, leading to membrane hyperpolarisation and relaxation. This occurs at least partly by mechanisms independent of adenylyl cyclase activity and cyclic AMP production and may involve the regulation of capacitative Ca^{2+} entry by small G proteins[38-41].

Long-term treatment with a receptor agonist often leads to receptor desensitization and a diminution of effect. The rate and degree of β_2 adrenergic receptor desensitization depend on the cell type. The β_2 receptors on human bronchial smooth muscle are relatively resistant to desensitisation, whereas receptors on mast cells and lymphocytes are desensitized rapidly following agonist exposure[42, 43]. This may help to explain why there is little evidence that these drugs are effective in inhibiting airway inflammation associated with asthma.

However, adding a long acting β_2 agonist to the inhaled corticosteroid regimen is often more effective in long-term control of asthma[43, 44].

- **Inhaled corticosteroids**

Inhaled corticosteroids are effective in controlling airway inflammation and are considered the preferred therapy for persistent asthma of all ages and disease severity[45]. Treatment with inhaled corticosteroids improves the control of the disease by decreasing inflammation in the bronchial epithelium and in the airways, decreasing airway hyper-reactivity and improving asthma symptoms and decreasing the frequency and severity of asthma exacerbations[46]. The anti-inflammatory effects of glucocorticoids in asthma include modulation of cytokine and chemokine production, inhibition of eicosanoid synthesis, marked inhibition of accumulation of basophils, eosinophils and other leukocytes in lung tissue, and decreased vascular permeability[46]. The profound and generalised anti-inflammatory actions of this class of drugs explain why they are currently the most effective drugs in treatment and control of asthma.

- **Leukotriene receptor inhibitor**

Leukotriene modifying drugs act either as competitive antagonist of leukotriene receptors or by inhibiting the synthesis of leukotrienes. Cysteinyl leukotrienes (Cys-LT) are potent constrictors of bronchial smooth muscle. On a molar basis, leukotriene D4 is nearly a 1000 times more potent as a bronchoconstrictor than histamine[47]. The cys-LT1 receptor is

responsible for the bronchoconstrictor effect of leukotrienes[48, 49]. Montelukast acts as selective high-affinity competitive antagonist to the cys-LT1 receptor[50, 51]. The effects of cys-LTs that are relevant to asthma are not limited to bronchial smooth muscle contraction. Cys-LTs can also increase micro vascular leakage, increase mucus production, and enhance eosinophil and basophil influx into the airways[52]. Therefore, inhibition of these non-smooth muscle effects of leukotrienes may also contribute to the therapeutic effects of montelukast.

Chapter 4

The Underlying Genetics Of Asthma

Asthma is a complex disorder resulting from interactions between multiple genes and environmental factors. However, the tools available to identify relatively modest effect sizes with contributions from multiple sources have been limited. The first study to successfully link a genomic locus (11q13) to atopic disease was not reported until 1989[53]. Since then, several candidate gene studies have been published so far. Unfortunately, results from many of these studies have not been replicated, even when apparently similar patient populations with similar selection criteria are used in subsequent analyses. The recent application of genome-wide association (GWA) studies to the analysis of allergy and asthma genetics have aided in combining the previously identified associations, and the biologic role and functional effects of variation of these candidate genes[54].

Studies of asthma genetics have raised new interest in the body's first line of immune defence, the epithelial barrier, in the pathogenesis of asthma. It is suggested that loss-of-function filaggrin gene variants are associated with asthma susceptibility and severity[22, 23]. Genes involved in mediating the response to inhaled bronchodilators appear to be important contributors to asthma severity. Variations in the *ADRB2* modulate the response to inhaled β agonist[55, 56]. The gene encoding the β_2 adrenergic receptor is an intronless gene that has been localised to q3 1q32 on chromosome 5[57, 58, 59]. This locus is close to the locus called

"5q cluster" in which the genes for IL-3, IL-4, IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF) are located[57, 58, 59].

Recent application of the GWA study design to asthma has produced much more robust results and is able to do so in a hypothesis-independent manner. Additional research is needed to identify additional causal genetic variants, better understand and gene–environment interactions, and explore the role of epigenetic phenomena in asthma susceptibility and severity in order to translate the findings to the clinical arena.

I have chosen two exemplar genes for the purpose of my work presented in the thesis. These genes were chosen on the basis of their significant functional roles in the context of the clinical outcomes which are most relevant in the day-to-day clinical practice, i.e. asthma exacerbations and effects on drug efficacy. The *FLG* null mutations could affect allergen entry and be important for susceptibility or early sensitization and thereby increasing the risk of asthma attacks. The *ADRB2* gene variations which affects drug efficacy and could affect asthma control including exacerbations. I have chosen these two genetic variations to explore their effects particularly on asthma exacerbations as this is common for both these genes.

Chapter 5

β_2 Adrenergic Receptor Gene Variations And Asthma

Retrospective genotype- stratified analyses suggest that polymorphic variations at the 16th amino acid residue of the β_2 adrenergic receptor is associated with adverse effects of β_2-agonist use in patients with asthma[60, 61]. Previous research has shown that there is an increased hazard for asthma exacerbations in children for the homozygous arginine-16 genotype (Arg16Arg) of the *ADRB2*, especially those on regular salmeterol[21]. It was found that, in the children receiving salmeterol, there was a 9 fold greater risk of school absences due to asthma in the Arg/Arg group in comparison to the Gly16 carriers[21]. In the cohort not receiving salmeterol, there was no evidence of any genotype-dependent increase in school absences due to asthma. The Arg16Arg children on salmeterol had a significantly increased risk of extended school absence of over 1week from asthma[21].

The Salmeterol Multicenter Asthma Research Trial (SMART) was conducted in United States during 1996- 2003 to compare respiratory- related and asthma- related outcomes in subjects receiving usual asthma pharmacotherapy alone or usual pharmacotherapy plus salmeterol[62, 63]. In the SMART study there were small but statistically significant increases in respiratory and asthma- related deaths and combined asthma-related death or life-threatening experiences in the total population in patients receiving salmeterol compared with placebo. It was also noted that the imbalance largely occurred in the African-

American subpopulation[62]. Whether this is due to factors including but not limited to a physiologic treatment effect, genetic factors, or patient level behaviours leading to poor outcome, remains unknown. However, it is hypothesized that a genetic variation in the β_2-adrenergic receptor may influence the response to β_2- agonist therapy, and the frequency of genetic variations in the β_2- adrenergic receptor gene are higher in subgroups such as African Americans compared with the overall population[64]. Analyses of previously published clinical trials on the long-term use of short acting β_2 agonist (salbutamol) have also showed that this hypothesis is correct for individuals homozygous for arginine (Arg/Arg genotype) at the 16th amino acid position of the β adrenergic receptor[65].

Phosphorylation of the receptor by β_2-adrenergic receptor kinase or other closely related G protein-coupled receptor kinases is the principal mechanism of homologous desensitization of the β_2-adrenergic receptor. Phosphorylation by protein kinase A and G protein-coupled receptor kinases desensitize β_2-adrenergic receptor signalling, and these mechanisms may be involved in cell and organ homeostasis and tolerance to agonists[66]. Significant cell-type variation in expression of β_2-adrenergic receptor kinase can be directly related to the extent of short-term agonist-promoted desensitization of the β_2-adrenergic receptor[67].

The 'on demand' use of inhaled short-acting β_2-agonists during asthma exacerbations is recommended by national guidelines and represents the cornerstone of asthma management worldwide[18, 68-70]. Previous research has shown that there is an increased risk for exacerbations in asthmatic children and young adults homozygous for the Arg16 variant of the Arg16Gly *ADRB2* genotypes [21]. In addition, the risk of possessing the Arg16 variation

was significant in asthmatics on regular inhaled long-acting β_2-agonists, with a gene/dosage effect for the Arg16 variant[21].

Irrespective of regular salmeterol use, asthmatics are routinely advised inhaled short-acting β_2-agonists on demand (e.g., before exercise, after allergen or cold air exposure or when they are developing exacerbations) [18, 69, 70]. This is a treatment strategy which is globally adopted in current guidelines and regularly scheduled use of salbutamol is no longer recommended[18, 35]. Several studies on older adults have suggested that homozygous Arg16 status is associated with reduced peak flows and increased exacerbations in asthmatics treated with regularly scheduled but not on demand short-acting β_2-agonists[63-65, 71]. However, the role of the Arg16 allele on exacerbations in steroid treated asthmatic children using 'on demand' short-acting β_2-agonists *per se* has not been assessed.

Further to the previous study[21], we have now doubled the size of the BREATHE cohort, aiming to address a number of additional questions, and have examined the association of the Arg16 variant with exacerbations upon exposure to both long and short acting β_2-agonists.

Chapter 6

Filaggrin Null Mutations In Childhood Asthma

Filaggrin is a highly abundant epidermal structural protein facilitating epidermal differentiation and skin barrier formation[72]. The *FLG* gene, located on human chromosome 1q21.3, encodes the giant (>400 KDa) polyprotein profilaggrin, which consists of 10-12 tandemly repeated filaggrin subunits[73]. Profilaggrin accumulates in dense granules within the keratinocytes of the stratum granulosum, the last living cell layers of the epidermis. Upon terminal differentiation of these cells to form the stratum corneum, the chemically modified, dead layers of the outermost epidermis, within which the skin barrier function resides, the inert profilaggrin molecule is proteolytically processed into multiple copies of active filaggrin. The liberated filaggrin aggregates the keratin cytoskeleton leading to cell compaction and squame formation. Enzymatic cross-linking of the protein and lipid components of the newly formed squames leads to formation of a chemically impermeable barrier whose function is to retain water and resist entry of antigens, allergens and irritants from the environment[72]. Disruption of barrier formation due to a reduction or complete absence of epidermal filaggrin expression has been postulated to lead to chronic transcutaneous antigen/ allergen/ irritant transfer, which via a TH2-mediated immune response, leads to atopic eczema and secondary allergic reactions, importantly including atopic asthma[22, 74].

Previous work has shown that polymorphic variation of the filaggrin gene is involved in regulating the overall burden of asthma[22, 23]. This occurs through an increase in asthma susceptibility[22] and also an increase in medication requirements[23] in children with established asthma. Weakening of physical barrier of skin in *FLG* deficient individuals may potentiate the effect of relevant environmental exposures. There is also evidence of significant interaction between the filaggrin loss-of-function mutations and cat exposure at birth on the development of early-life eczema[75].

Two independent mutations in the gene encoding filaggrin (*FLG*; R501X and 2282del4), carried by about 9% of people of European origin, result in the loss of processed functional filaggrin in the epidermis[22, 76]. These genetic mutations, previously proven to impair the formation of stratum corneum[76], strongly predispose to childhood eczema in several white European populations, where these mutations are prevalent[22, 77-82], including Scottish, English, Irish, Danish and German populations. Analogous mutations leading to loss of function have been recently reported to be significantly associated with atopic dermatitis and ichthyosis vulgaris in the Japanese population[83] and may even predict more severe and persistent forms of atopy[78]. This gene may contribute to atopic disease burden to varying degrees worldwide. Recently, the genetic architecture of filaggrin-related atopy has been shown to consist of a combination of a small number of prevalent null mutations as well as several rare or family-specific mutations[73], as recently reviewed[84].

The combined genotype of the two most prevalent filaggrin variants in Europeans, R501X and 2282del4, was the focus of the previous work from our group[22]. However, further work

suggested that the R501X mutation may have greater penetrance in determining higher serum IgE levels in patients with atopic eczema in comparison to those carrying the 2282del4 mutation[82]. As similar penetrance differences may occur in asthma, the relative effects of the null mutations as well as the combined genotype, on the asthma severity outcomes and symptomatic control measures of the BREATHE study, require to be compared. The previous data had demonstrated that individuals with *FLG* null alleles have a significantly increased disease burden, both in terms of lung function, the null mutation carriers having greater airway obstruction, and in the intensity of medication required for disease control[23]. The individual contribution to the overall signal of the 2282del4 allele was lower than that observed for the R501X mutation. However the association of these mutations with the risk of asthma exacerbations has never been assessed. In asthmatic children, asthma-related school absences[17], requirement of oral steroids[18, 19] and hospital admissions[20] represent well validated measures of asthma exacerbations. A combined score has been developed by our group, involving yes/no responses for any of the above three measures of exacerbations over a 6 month period of reporting, to explore asthma exacerbation risk from PPARγ genotype variation[24]. Here I have used this score to compare the relative effects of the two filaggrin mutations and the combined genotype on the risk of asthma exacerbations. I also explored in this study the relative penetrance of the 2 mutations and the combined genotype on a larger asthmatic population.

Chapter 7

Epithelial Permeability And Filaggrin

The filaggrin protein present in the stratum corneum is thought to maintain skin barrier integrity in at least 2 ways. Firstly, it arranges the keratin filaments into structural scaffolds and secondly it osmotically maintains skin hydration[72, 85, 86]. It is now known that children with common (approximately 9% of the population) loss-of-function mutations in this protein are more likely to suffer from early-onset eczema, asthma and other allergies such as to grass pollen, cat and house dust mite[22, 75]. Interestingly, filaggrin is not expressed in the bronchial mucosa[87], which has led others to suggest that asthma susceptibility in patients with loss-of-function *FLG* variants may be due to allergic sensitization that occurs as a result of a weakened epidermal barrier[88].

Filaggrin is essential for the cell compaction process preceding chemical cross-linking during the genesis of the stratum corneum. Therefore, it is a key molecule in the initiation and maintenance of skin barrier function[89]. The terminal degradation products of filaggrin may also act as a ‘‘natural moisturizing substance’’[82]. FLG mutations are an important predisposing factor for atopic dermatitis and secondary atopic phenotypes like asthma, suggests that the skin barrier defect is a primary key event leading to allergic sensitization and development of atopic phenotypes[90].

Through literature searching I sought to identify a technique that could measure the differences in epidermal permeability between children carrying filaggrin gene defects and controls. Transepidermal water loss (TEWL) and stratum corneum hydration, which are measurements of skin barrier function, were reported to increase in patients with atopic dermatitis due to their skin barrier insufficiency[91, 92]. Significant correlations were observed between penetration rates of a hydrophilic dye and elevated IgE levels in patients with severe atopic dermatitis[93]. Skin barrier function could be evaluated by measurements of TEWL, stratum corneum hydration, and thickness that are useful markers of skin barrier function[94]. In ichthyosis vulgaris, the epidermal barrier function measured by TEWL is known to be associated with the severity of the disease[95]. Additionally, in patients with atopic dermatitis, TEWL was increased, although controversy still remains as to whether the defective barrier function in atopic dermatitis patients is a primary cause of atopic dermatitis or a secondary consequence following dermatitis[96].

Transepidermal water loss represents the outward diffusion of water through skin and its measurements are used to gauge skin barrier function[97]. Readings increase when the integrity of the stratum corneum barrier is compromised, and they correlate with percutaneous absorption[98]. Few studies have characterized TEWL[99] in young children with atopic dermatitis, and studies in children have not compared altered epithelial permeability in children with filaggrin defects versus the normal population.

The three possible routes of allergen entry in humans resulting in atopic diseases are the skin, respiratory tract and gastro-intestinal tract. A previous study has identified the

presence of filaggrin in the human hard palate and buccal mucosa, but not in the nasal (inferior turbinate) mucosa, or in the oesophagus (proximal, mid or distal) [100].

In routine clinical practice it has been observed, that the removal of the food allergen from the patient's diet leads to a significant clinical improvement in children with atopic dermatitis. A possible hypothesis is that the IgE-bearing dendritic cells help in the circulation of allergen from the gastro-intestinal tract to the skin and activate local T cells to result in atopic diseases. However, simultaneous evaluation of epidermal barrier impairment measured using a tewameter, and the risk of allergen sensitization by measuring the permeability of the gastro-intestinal tract, has not yet been performed in children.

I performed a pilot study to noninvasively explore the permeability changes in the skin and the intestine, in order to help identify the primary site of allergen entry in humans.

SECTION III

METHODS

The BREATHE study was approved by the Tayside Committee on Medical Research and Ethics. Informed consent was provided by the patient and parent/ guardian as relevant, in compliance with all principles of the Helsinki Accord[101-103].

Chapter 1

Personal Interview

Clinical and genotype information were collected from 1182 children and young adults with physician diagnosed asthma for the BREATHE study using a simple questionnaire (figures 3.1.1 and 3.1.2). The dataset includes information about demographic, anthropometric and clinical details from 1182 individuals attending primary and secondary clinics in Tayside and Dumfries, Scotland, from 2004 to 2008 (age 3-22 years). The data was collected on 546 children previously and has now been extended to 1182 of which I collected data from 411 participants, working with Ms Inez Murrie.

All asthma patients were recruited through 29 primary care practices and 2 secondary care asthma clinics. Asthma was diagnosed by the physicians according to the Scottish Intercollegiate Guidelines Network/British Thoracic Society Diagnostic Guidelines[18].

The clinical patient data for the study was collected through the paediatric wards and asthma clinics of Ninewells Hospital and Perth Royal Infirmary, Tayside, or through pre-arranged visits to primary care. An initial search was carried out to identify patients and the parents and patients were contacted as appropriate. History and baseline clinical data were collected. The diagnosis of asthma, and allergy test results were obtained from the medical records, while the rest of the data were obtained from parental recall. Saliva samples were collected for genotyping and archiving. All data were anonymised. Each salivary sample was coded with a unique number, not the child's name or hospital identification number. These were stored completely separately. No computer file contains both names and study numbers, and all study information is identified by the study numbers.

The patients were seen in the asthma clinic setting, where a detailed personal and family history was obtained including detailed quantified information on school absences, courses of oral steroids and hospital admissions over the previous 6 months. In addition, parents were asked, ''has your child ever had eczema or an itchy rash?'' For the assessment of seasonal rhinitis, the parents were asked, "does your child have hayfever or does he/she sneezes more in a particular time of the year?" "Does your child have runny nose most of the time of the year?" was asked to assess for perennial rhinitis. The asthma prescribing level was determined as the modified BTS guidelines for physician-led management of asthma, as follows: step 0 – no use of inhaled salbutamol on demand within the past month; step 1 - inhaled short-acting β_2 -agonists eg. salbutamol on demand; step 2 - regular inhaled

steroids plus inhaled salbutamol on demand; step 3 - regular inhaled long-acting β_2 - agonists eg. salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand. From this data a global index of asthma severity was derived through construction of a composite variable, as reported previously[24]. Steps 0 and 1 were considered as mild asthma while steps 2, 3 and 4 were included in the moderate to severe group. The changes in medication within the previous 6 months were recorded as well.

Family history for asthma, eczema and allergic rhinitis were explored and expressed as present or absent. Asthma related school absences[17] (days off work/university for young adults) use of short courses of oral steroids[18, 19] and admission to the hospital[20] due to severity of asthma (minimum once over the previous 6 months) were grouped as present or absent. The total asthma exacerbation response was defined as the presence or absence of any of these measures during this period of time. This was again grouped as present or absent to create an asthma exacerbation yes/no response that has been validated as a measure of asthma severity through several publications[21, 23-26, 104, 105]. Although the individual measures for asthma exacerbations have been used for assessment of exacerbation of asthma in children and young adults[17-20], the composite exacerbations scoring system has not yet been externally validated.

Figure 3.1.1: The BREATHE study questionnaire page 1

BREATHE STUDY
(Children's Genotype Project)

Name_____ DOB/CHI____/___/_____
Address_____ Occupation_____
_____ Telephone Number_____
_____ Mobile Number_____
Name of Parent_____ Email_____
Mother's DOB_____ Father's DOB_____
GP_____ Health Centre_____
Height (cms)_____ Weight (Kgs)_____
Date & Source_____ Date Data Entered_____

History
Does the child have: Eczema ☐ Perennial rhinitis ☐ Seasonal rhinitis ☐
Does anyone else in the family have:

	Mother	Father	Siblings	Other	None
Asthma	☐	☐	☐	☐	☐
Eczema	☐	☐	☐	☐	☐
Allergic rhinitis	☐	☐	☐	☐	☐

Does the child have any allergies?

Agent	Reaction

Known triggers for asthma?

Does the child/ young adult smoke? Yes No
If yes, how many per day? _____

Is the child exposed to smoking?

At home ☐ With Relatives ☐ With Childminder ☐ Other ☐
Not exposed ☐

Figure 3.1.2: The BREATHE Study questionnaire page 2

Is the child exposed to animals? Cat ☐ Dog ☐ Horse ☐ Bird ☐
Other☐ (Specify)_____ Not exposed ☐

Has the child ever had an allergy test? Yes ☐ (SPT ☐/RAST ☐) No ☐
Results:_____
_____

Current Medication

Drug	Device	Dose per puff	Frequency

Inhaler technique Good ☐ Poor ☐ Not applicable ☐

Bronchodilator use in the last 6 months: None ☐ Occasional ☐ Daily ☐ Excessive ☐
Comments_____

Courses of oral steroids in the last 6 months_____

Number of hospital admissions in the last 6 months_____

Absence from school/ nursery/ college/ work?
None ☐ 1-2 days ☐ Up to 1 week ☐ > 1 week ☐

Lung Function

	PEFR	FEV_1	FVC	FEV_1/FVC
Predicted				
Actual				
% Predicted				
Reversibility				
Actual (post β_2)				
% Change				

Other Information_____
_____
_____

Chapter 2

Pulmonary Function Tests

Pulmonary function tests were performed using the Microlab ML3500 MK8 portable Spirometer[106, 107] (Micromed, Rochester, United Kingdom). It uses a stable form of transducer known as a digital volume transducer. This measures the expired air directly at body temperature and pressures with saturated water vapour and avoids the inaccuracies of temperature corrections. The transducer is insensitive to the effects of condensation and temperature. It was used to measure the forced spirometry to measure forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), forced expiratory flow 25% to 75% (FEF_{25-75}) and peak expiratory flow (PEF). The ratio of forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) constitutes an established index of airway obstruction[108]. This ratio was used as the primary measure of airway obstruction for the study. All the values were corrected for age, sex and height of the participant and the percentage predicted values were taken into account. The calibration of the machine was routinely done by a 3litre syringe connected to the transducer. A calibration error of ±3% was considered acceptable as per the American Thoracic Society/ European Respiratory Society recommendations[106].

The subjects were instructed to take a deep breath in and exhale as forcefully and long as possible into the mouthpiece attached to the transducer so as to empty their lungs. At least 3 measurements were taken to calculate a mean for each individual. The results were printed on thermo stable printer paper.

The pulmonary function data were collected at a single visit. A minimum of 3 results within 10% of each other was recorded, and the result with the highest FEV_1 was analyzed. The participants were not suffering from asthma exacerbations or other acute illnesses at the time of the measurement of pulmonary function. The lung function test results were expressed as a percentage of that predicted using the data of Rosenthal et al[107].

Chapter 3

Saliva Collection For DNA and Genotyping

All DNA samples were collected from the saliva with informed consent[101-103]. The saliva was collected by two methods.

- **Mouthwash method**

It was ensured that the participant has not eaten or cleaned their teeth immediately before taking the sample. The participant was encouraged to take 4-5mls of clean tap water into his/ her mouth and swirl around the mouth for approximately 20-30 seconds. The sample was then spit back into a universal container. The container was sealed correctly and labelled with the study identification number, not the patient's name, and that the correct patient details were associated with the identification number. This technique was very easy to perform, did not require any special expertise and was child-friendly.

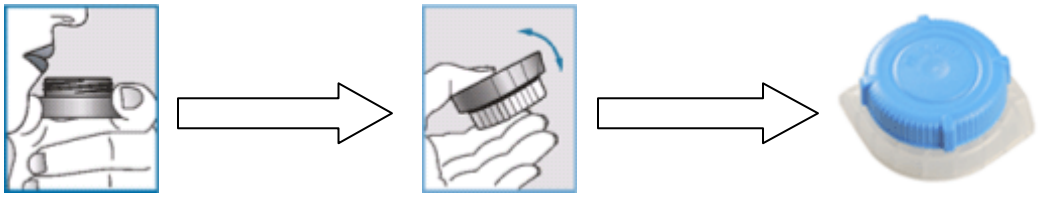
- **Saliva collection by Oragene® DNA collection kit**

Oragene® DNA is a non-invasive DNA collection kit that is used by untrained study participants including children and elderly (http://www.dnagenotek.com). The median DNA yield from 2ml Oragene® saliva samples is about 110 microgram. This kit was used for DNA yield, preservation and purification. It has low bacterial content and is equivalent to DNA from blood for downstream applications. The preservation and storage of DNA samples was of immense importance for this study. The DNA from saliva is stable in this kit for up to 5 years in room temperature. The stability is achieved with proprietary reagents that inactivate bacteria and nucleases in saliva and minimize chemical hydrolysis of DNA.

Figure3.3.1: Oragene® DNA collection kit

Children over 5 years of age were encouraged to have a drink of water prior to providing the sample. After a few minutes they were requested to spit in the container directly, up to the mark. The drink prior to the procedure would have enhanced the production of saliva. Then the container was tightly closed so that the solvent came to contact with the sample. This allowed preservation of the sample in room temperature.

Figure 3.3.2: Method of collection of saliva in the Oragene® DNA collection kit in children older than 5 years of age



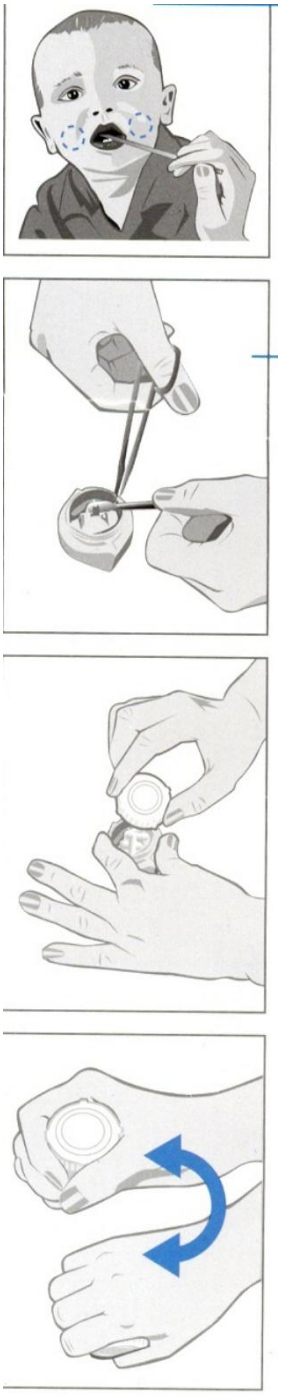
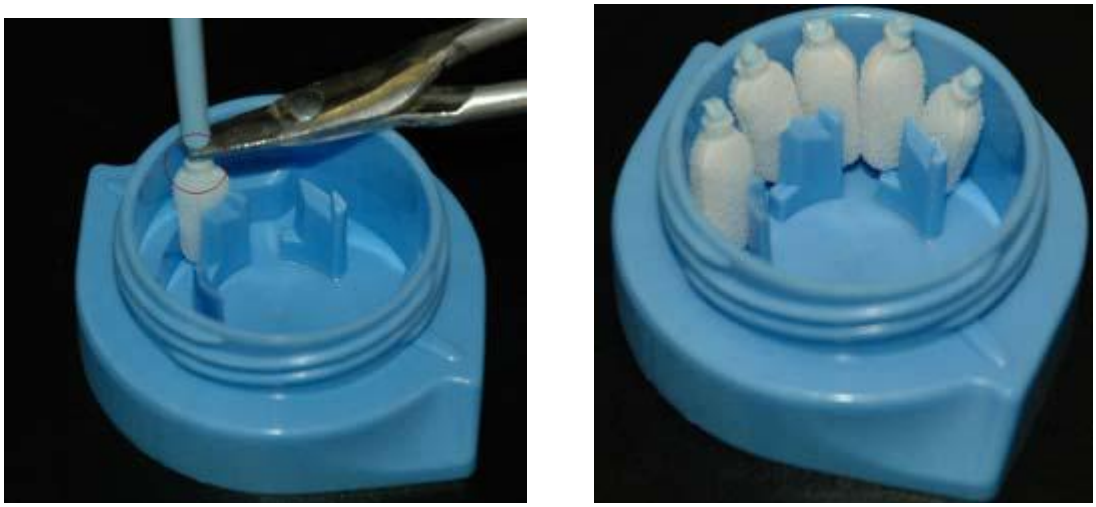


Figure 3.3.3: Collection of saliva by the Oragene®® DNA collection kit in a child under 5years of age.

In younger children, a saliva sponge was placed in the child's mouth in the space between the gums and the inner cheek. The saliva sponge was gently moved around the upper and lower cheek pouches on both sides of the mouth to soak up as much as saliva as possible. The sponge was left in the child's mouth for about 60seconds.

Once collected, the sponge was cut into the blue base of the Oragene® kit as shown in figure. The sponge was then placed firmly against the bottom of the kit between the tooth and the kit wall. This action ensured that the sponge tip remains in the container during the cutting action. Using the scissors provided, the narrow part of the handle was cut just above the sponge.

Figure 3.3.4: Method of cutting the saliva sponge and placing in the kit



For the collection of up to five saliva sponges, these steps were followed at 5 minute intervals. The kit was then carefully capped and tightened firmly. The Oragene® liquid released from the cap preserved the DNA collected by the sponges. The samples were then sent in batches to the Biomedical Research Institute, University of Dundee, for genotyping.

Genotyping

The genotyping was done at the Biomedical Research Institute, University of Dundee. I have observed the methods of genotyping being performed by Dr Roger Tavendale in Professor Colin Palmer's laboratory.

DNA was prepared using the Qiagen Dneasy 96 kit, and genotypes were determined using Taqman-based allelic discrimination assays on an ABI 7700 sequence detection system[114]. The DNA was archived for future research.

- **FLG R501X and 2282del4**

All primers and probe sequences have been shown in previous publication[22]. Mutation R501X creates a new NlaIII restriction enzyme site, and 2282del4 creates a new DraIII site, which were used to screen short, highly specific PCR fragments for these variants, as described previously[22]. Genotyping for R501X was also performed using a TaqMan-based allelic discrimination assay (Applied Biosystems). Standard procedures were used based on Applied Biosystems reagents and 10-µl reaction volumes. Allelic discrimination was assessed using an Applied Biosystems 7700 sequence detection system. Mutation 2282del4 was also genotyped by sizing a fluorescently labelled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer. Ten-micro litre PCR reactions were carried out using primers DEL4.F2 and DEL4.R1 in AmpliTaq Gold buffer containing 1.5 mM MgCl2

(Applied Biosystems), 10 nmol of each dNTP and 1 unit AmpliTaq Gold DNA polymerase. Reactions were amplified as follows: 94ºC (12 min), one cycle; 94 ºC (15 s), 58 ºC (30 s) and 72 ºC (45 s), 30 cycles; and 72 ºC (5min), one cycle. Fragments were diluted 1:60 and sized against ROX-500 size markers according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 199 bp, and the 2282del4 allele was 195 bp.

AA refers to the wild- type FLG genotype for R501X and 2282del4 mutations, Aa refers to heterozygous genotype for either R501X or 2282del4, and aa refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype. The homozygous, heterozygous and compound heterozygous genotypes were considered together as Aa/aa.

- **Gly16Arg and Glu27Gln**

DNA was prepared using the Qiagen Dneasy 96 kit, and genotypes were determined using Taqman based allelic discrimination assays on an ABI 7700 sequence detection system[114].
For the Gly16Arg variant, the following probes and primers were used:
forward primer: GAACGGCAGCGCCTTCT;
reverse primer: GCACATTGCCAAACACGATG;
Arg16 probe: Cal Orange or VIC-CACCCAATAGAAGCCATGCGCCGGACCACGAC-BHQ; Gly16 probe: FAM-CACCCAATGGAAGCCATGCGCCGGACCACGAC-BHQ.

For the Glu27Gln variant the forward primer was identical to the forward primer for the Gly16Arg variant.

reverse primer: TGAGAGACATGACGATGCCC.

Gln27 probe: Cal Orange-or VIC-CCATGCGCCGGACCACGACGTCACGCAGCAAAGGGACGA-BHQ;

Glu27 probe: FAM-CCATGCGCCGGACCACGACGTCACGCAGGAAAGGGACGA-BHQ.

Chapter 4

Measurement of Transepidermal Water Loss

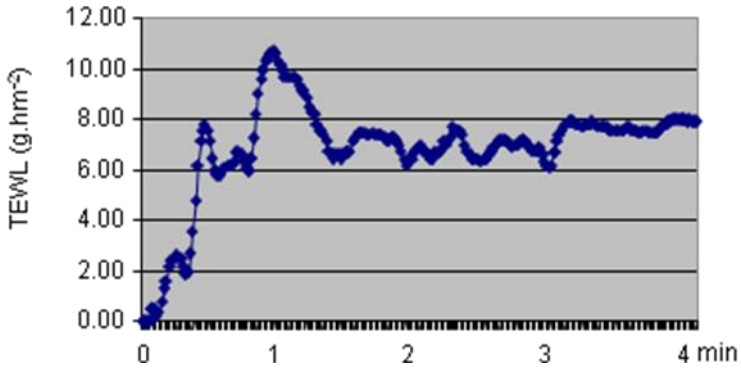
Skin hydration and integrity, as measured by transepidermal water loss, are important parameters for objectively quantifying atopic dermatitis in children. Transepidermal water loss was measured by a non-invasive technique using Tewameter (CK electronic GmbH) [109-111] website: www.courage-khazaka.de). The measurement of the water evaporation is based on the diffusion principle in an open chamber $dm/dt = D\ X\ A\ dp/dx;$ where A= surface in meter^2, m=water transported (in gram), t= time (hours), D= diffusion constant, p= vapour pressure of the atmosphere (mm Hg), x= distance from skin surface to the point of measurement (meter) (www.courage-khazaka.de). The density gradient is measured indirectly by the two pairs of sensors (temperature and relative humidity) inside the hollow cylinder and is analysed by a microprocessor. The small size of the probe head minimizes the influence of air turbulences inside the probe. Also the low weight of the probe has no influence on the skin surface structure and allows easy handling. All the calibration data are inside the probe. Therefore the probe is self-contained.

The measuring principle is based on the open chamber measurement. With this method, continuous measurements are possible without any influence on the skin surface. For stable measurements the Probe Heater PR100 keeps the probe head to a certain temperature of 28-32^{o}C (corresponding the skin temperature).

I placed the probe on the participants forearm, midway between the wrist and elbow. The recording of measurement was represented graphically on the computer. Measurements were allowed to stabilise for around 5 minutes before recordings were made for a 2 minute period, and the mean rate of water loss calculated for the 2 minute time period. Measurements were made at least 3 times in total for each participant and an overall mean transepidermal water loss calculated. The skin permeability of one child was higher compared to the others. To maintain consistency in the operating procedure, the measurement was not repeated, however, the participant was excluded from the final analysis.

Figure 3.4.1: Tewameter and the probe

Figure: 3.4.2: Graphical representation of the transepidermal water loss measurement



Chapter 5

Dual Sugar Test

Dual sugar absorption test is a non-invasive test of intestinal permeability and has been used to assess the permeability of the small bowel mucosa, in a variety of intestinal diseases. In these tests, the permeability to nonmetabolizable mono- and disaccharides like mannitol/ lactulose or rhamnose/cellobiose is compared and expressed as the ratio of the concentrations of these saccharides in a timed urine sample[112]. Lactulose/mannitol excretion ratios have been proposed as a useful, non-invasive test for screening and monitoring celiac disease[112]. Following overnight fast, the participants drank a solution containing lactulose, a non-metabolised, high molecular weight sugar (0.1g/kg body weight) and mannitol, a readily absorbed, low molecular weight sugar (0.1g/kg body weight) (volume drunk= 1ml/kg of body weight). The participants did not have any food or drink for two further hours. They were then given 500 mls of water to drink. All urine produced during the 5 hour period following ingestion of the sugar solution was collected in a container with 10mg of gentamicin, to prevent bacterial consumption of any excreted sugar. The total volume of urine was measured and a small aliquot (3-5 mls) further sample was frozen.

The content of the sugars lactulose and mannitol were measured using high pressure anion exchange chromatography with pulsed amperometric detection[112-113]. This assay was performed by Dr Simon Fleming (Clinical Biochemistry, Royal Cornwall Hospital).

Chapter 6

Statistical Analyses

I have used SPSS for Windows versions 14-16 (SPSS Inc, Chicago, Ill) for all the statistical analyses.

- **β_2 adrenoceptor genotype variation and asthma exacerbations**

The dataset used for this analysis includes information about demographic, anthropometric and clinical details from 1182 individuals attending 29 primary care practices and 2 secondary care asthma clinics in Tayside, Scotland, from 2004- 2008 (age 3-22 years). A detailed history was obtained including information on school absences, usage of oral steroids and hospital admissions over the previous 6 months. For simplicity and greater accuracy through recall, only yes/ no responses for any of the three options were used for analysis.

Binary logistic regression was used to calculate odds ratios and P values for asthma exacerbations. To calculate the odds ratios (ORs) for comparison of risk, measures for asthma exacerbations were grouped according to severity. School absences, intake of oral steroids and admission to the hospital due to severity of asthma were grouped as present (minimum once over the previous 6 months) or absent. The total asthma exacerbation

response was calculated as any of these measures during the same period of time. This was again grouped as present or absent.

In order to explore the associations, I used a co-dominant model 0=Gly16Gly, 1=Gly/Arg16, 2=Arg16Arg, as has been previously examined[21]. Age, gender, and exposure to tobacco smoke were included in all models as covariates after step-wise removal procedures (covariates with $p<0.2$ retained). Seasonality, another potential covariate, did not contribute significantly to the model ($p>0.4$) and was not associated with genotype in any subgroup tested and hence was excluded from the final analysis.

I tested the effect of the Arg16 allele on asthma exacerbations in relation to inhaled β_2-agonist use from a number of different perspectives. Firstly, I tested the overall effect of the Arg16 allele on the individual measures of asthma exacerbations and on the overall risk of exacerbations. Secondly, I also tested the association of the genotype of *ADRB2* and exacerbations over asthma treatment steps 0-4 and in participants using regular salmeterol versus those not using regular salmeterol, in order to identify any treatment steps where the effect could be more prominent than others. Finally, I tested the hypothesis that there is an interaction between genotype and the daily use of any inhaled β_2-agonist (as required salbutamol and/or regular salmeterol) on the risk of asthma exacerbations. I also tested the association of Glu27Gln genotype with exacerbations conditioned on Arg16Gly polymorphic variation. Significance was predetermined at $p<0.05$.

- ***FLG* null mutations and asthma exacerbation study**

The dataset includes information about demographic, anthropometric and clinical details from 1135 individuals attending primary and secondary clinics in 29 primary care practices and 2 secondary care asthma clinics in Tayside and Dumfries, Scotland, from 2004 to 2007 (age 3-22 years).

To calculate the ORs for comparison of risk, measures for asthma exacerbations were grouped according to severity. School absences, intake of oral steroids and admission to the hospital due to severity of asthma were grouped as present (minimum once over the previous 6 months) or absent. The total asthma exacerbation response was calculated as any of these measures during the same period of time. This was again grouped as present or absent. Chi-square test was used to compare the effects of the mutations on total asthma exacerbations as well as its constituent measures. Significance was assessed at $p < 0.05$. Both one-tailed and two tailed *p*-values are shown due to the predictable nature of the direction of effect of the variants of the traits under test.

- **Epithelial permeability in *FLG* null mutations- A pilot project**

10 participants, heterozygous for the filaggrin null alleles, were recruited from the BREATHE study. The 14 control participants were volunteers who were not genotyped and did not have asthma or eczema. The median values for the skin and intestinal permeability tests were compared using the Mann Whitney test. A *p* value below 0.05 was considered significant.

SECTION IV

RESULTS

Chapter 1

Description Of the BREATHE Cohort

The population characteristics are fairly typical of young individuals with well controlled asthma derived from both primary and secondary care[115] (Table4.1.1). 1182 participants were Caucasian and the data from other ethnic groups were not sufficient for further analysis. The mean age for the study children was 10.3 ±4.05 years. 58.8% of the children were boys and 41.2% were girls. Mean body mass index (BMI) for the study children was 19.19± 4.32. According to the modified British Thoracic Society guidelines for treatment of asthma, 17.5% children were on step 1, 56% on step 2, 13.3% on step 3 and 10% were on step 4.

Overall prevalence of asthma exacerbations over the previous 6 months was 38.2%. The individual measures for exacerbations were also explored. 32.1% children needed days off school in the previous 6 months, 21.7% required a course of oral steroid and 13.1% children were admitted to hospital due to exacerbation of their asthma.

The family history of asthma, eczema and allergic rhinitis were explored and described in table 4.1.2. There was positive maternal history of asthma in 24.2%, eczema in 14% and allergic rhinitis in 25.5%. Positive paternal history for asthma was present in 19.4%, eczema in 7% and allergic rhinitis in 15.6%. Siblings of these children and young adults suffered from asthma in 30.2%, eczema in 21.1% and allergic rhinitis in 16.3%.

Table 4.1.3 describes the personal history of the participants as obtained during the interview. 52.6% children suffered from eczema. 21.2% suffered from perennial rhinitis and 23.4% suffered from seasonal rhinitis. 33.3% children were exposed to cigarette smoke and 62.3% were exposed to animals. Figure 4.1.1 describes the common domestic animals the children were exposed to.

Figure 4.1.2 describes the common asthma medications used in the participants. The commonest medication used for control and management of asthma was beclomethasone dipropionate (53%). Seretide (salmeterol with fluticasone) (16%) was the second most commonly used drug in children with asthma for their long term treatment. Montelukast was used by 11% of the participants.

Figure 4.1.3 describes the common allergens responsible for the atopy in the participants. It was noted that the common allergens responsible for atopic diseases were cat fur (20%), house dust mite (20%), dog hair (16%) and grass (16%). This was observed on the result of the skin prick test performed on the child in the past.

Table: 4.1.1: Demographic and clinical characteristics of participants for the BREATHE study (n=1182)

Mean (SD) age (years)	10.34 (4.0); (<18=1137; ≥18= 45)
Sex (Male/Female)	703/ 479 (58.8%/41.2%)
Mean (SD) body mass index	19.13 (4.5)
Pulmonary function (n=915)¶	
Mean (SD) PEFR (% of mean predicted)	87.66 (17.8)
Mean (SD) FEV1 (% of mean predicted)	95.83 (15.5)
Mean (SD) FVC (% of mean predicted)	92.20 (14.4)
Mean (SD) FEV1/FVC (% of mean predicted)	102.39 (9.7)
Modified British Thoracic Society (BTS) step of asthma treatment*	0= 39(3.3%); 1= 204 (17.3%); 2= 660 (56%); 3= 157 (13.3%); 4= 117 (10%)
Inhaled bronchodilator use†	0= 137 (11.6%); 1= 827 (70%); 2= 186(15.7%); 3= 31(2.6%)
School absences over previous 6 months	382(32.3%)
Courses of oral steroids over previous 6 months	263 (22.3%)
Hospital admissions over previous 6 months	157 (13.3%)
Overall asthma exacerbations over previous 6 months‡	456 (38.6%)

KEY: SD: standard deviation; PEFR: Peak expiratory flow rate; FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity; *Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand; †Inhaled bronchodilator use: 0= none, 1= occasional (more than once a week and less than daily use), 2= daily (200 mcg/ day required for symptom control), 3= excessive use (use of more than one dose of 200 micrograms/ day for symptom control); ‡Defined as any one of the following in previous 6 months: school absences, courses of oral steroids, or hospital admissions

Table: 4.1.2: Family history of asthma, eczema and allergic rhinitis in the study population (n=1182)

	Asthma	**Eczema**	**Allergic rhinitis**
Maternal	283 (23.9%)	161 (13.6%)	305 (25.8%)
Paternal	231(19.5%)	85 (7.2%)	188(15.9%)
Siblings	358 (30.3%)	249 (21.1%)	194 (16.4%)
Other	567 (47.9%)	279 (23.6%)	207 (17.5%)

Table: 4.1.3: Personal history of the participants of the BREATHE study (n=1182)

Eczema (%)	607 (51.4%)
Perennial rhinitis (%)	255 (21.6%)
Seasonal rhinitis (%)	279 (23.45)
Smoking (%)	24/ 1158 (2.0%)
Exposure to smoke (%)	394/ 750 (33.3%)
Exposure to animals (%)	736/ 446 (62.3%)
Triggers for asthma exacerbations as reported by parents	
Cold	386 (32.7%)
Exercise	445 (37.6%)
Viral illness	626 (53.0%)
Others	402 (34.0%)

Figure 4.1.1: Pie chart illustrating the exposure to animals

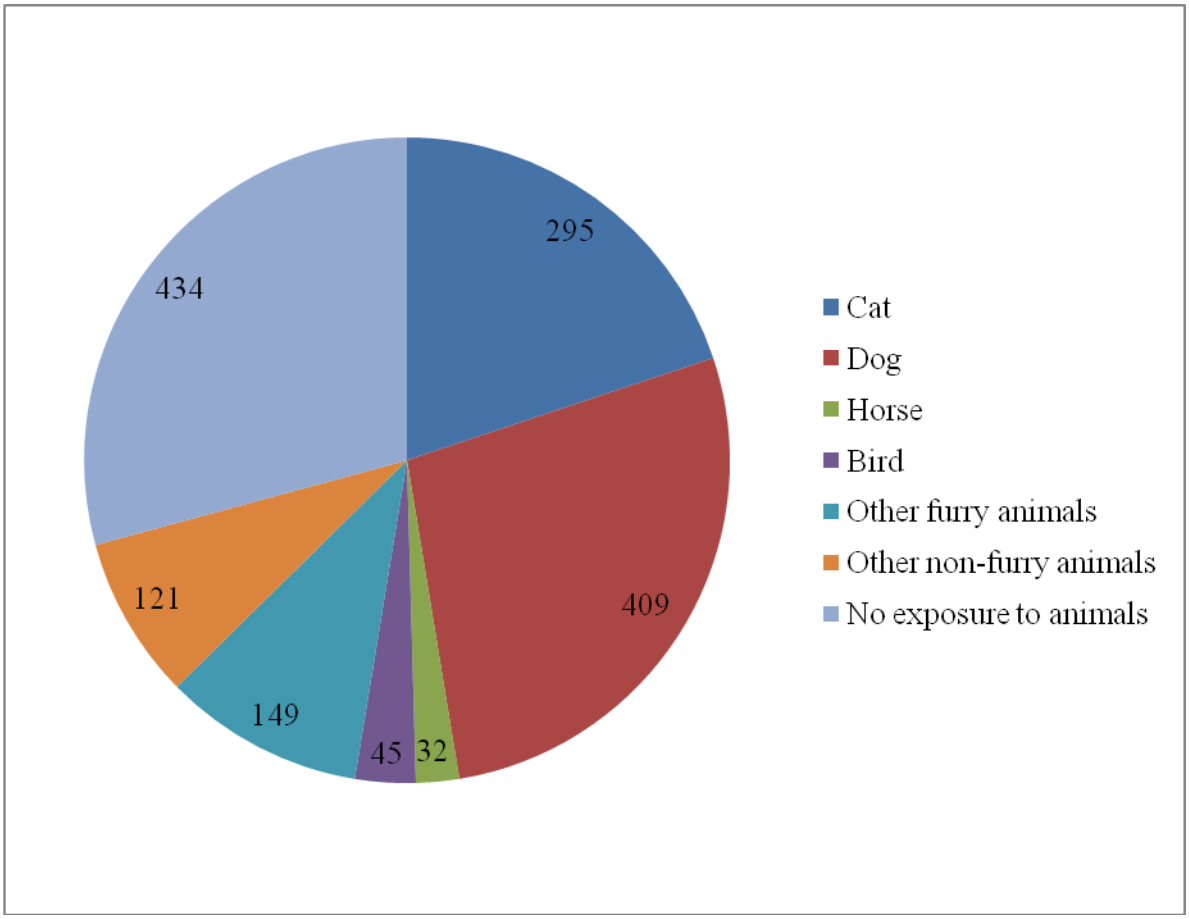


Figure 4.1.2: Medications prescribed for asthma control in Tayside and Dumfries (n=1182)

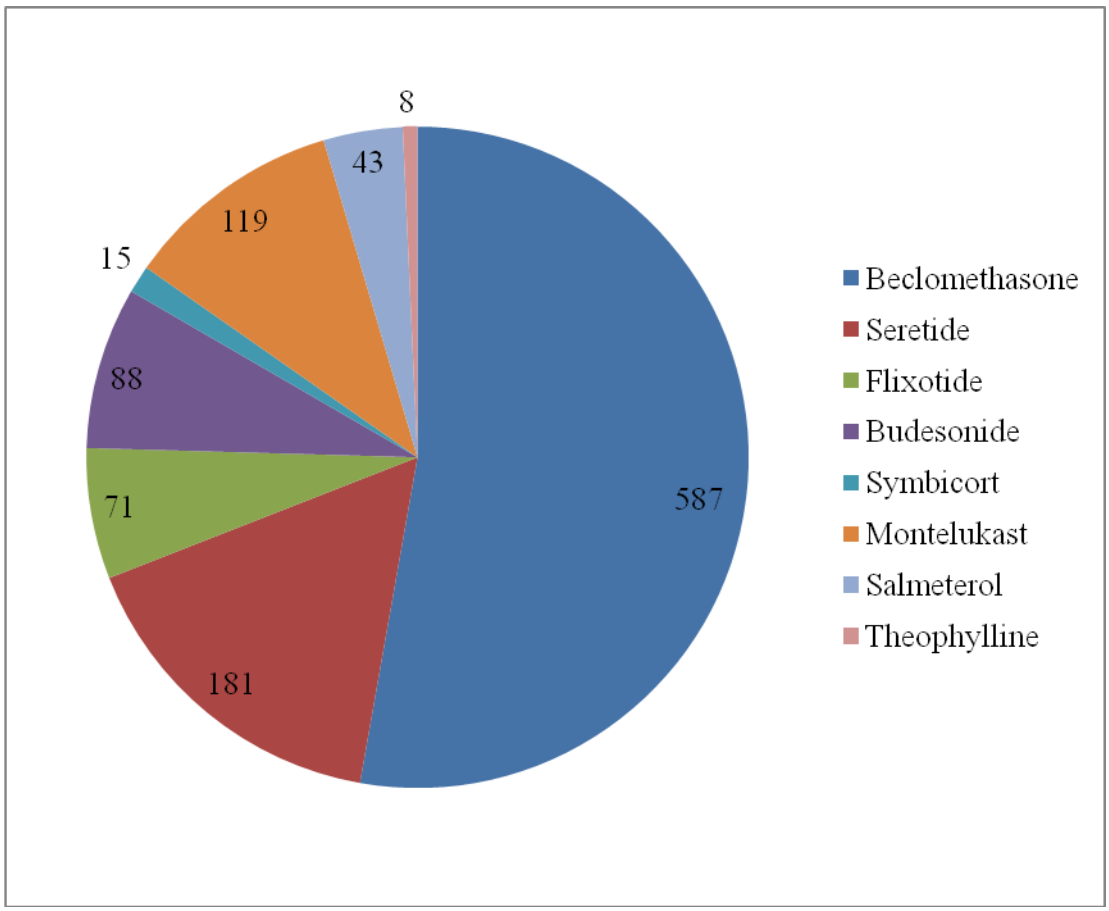
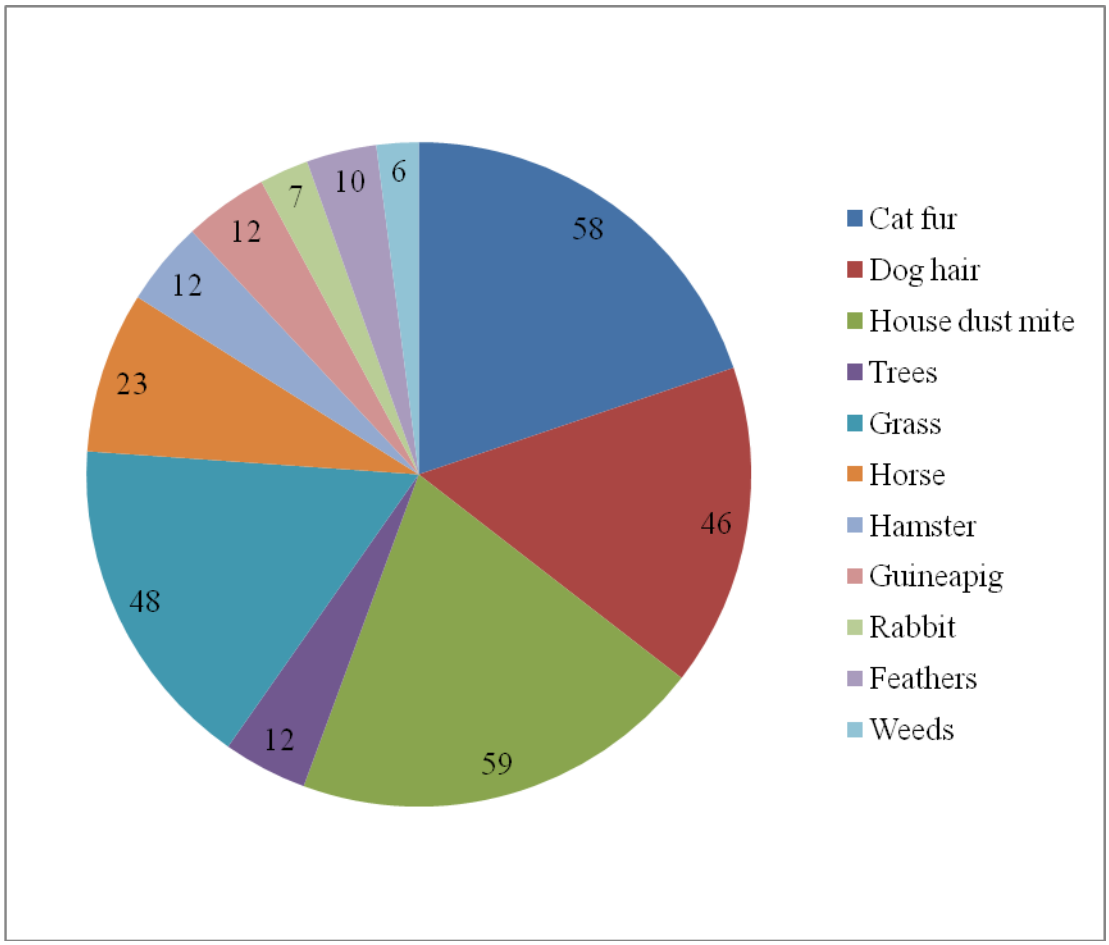


Figure 4.1.3: Common allergens positive on skin prick test (n=136)



KEY: 136 out of 1182 participants had skin prick test done as part of clinic work-up

Chapter 2

Effects Of β_2-adrenergic Receptor Gene Variations in Asthma Exacerbations

The population characteristics are described in details in table 4.2.1. Table 4.2.2 describes the medication use and episodes of exacerbations of asthma in the study participants. The prevalence of the Arg16Arg genotype was 15.3%, Arg16Gly 43.8% and Gly16Gly 40.8% (table 4.2.1) in children and young people with asthma within Tayside (Minor allele frequency= 0.37) (table 4.2.3). The minor allele frequency (MAF) was similar to that observed for the US and UK populations[21, 65, 116] (MAF 0.35 and 0.36 respectively) [64,117].

An increased risk per copy of the Arg16 allele, of requirement of short courses of oral steroids due to asthma exacerbations was observed (OR: 1.27; 95% CI=1.04-1.56 (p= 0.02)) (table 4.2.4). Table 4.2.5 describes the overall effect of the Arg16Gly genotype in the increase in school absences due to asthma exacerbations in children with asthma, over the previous 6 months. No increase in the risk of hospital admission due to asthma exacerbations was observed in the children with Arg16Arg genotype (table 4.2.6).

There was a significant increase in the overall asthma exacerbations (OR 1.30; 95% CI 1.09-1.55 (p= 0.003)) in children with asthma regardless of the asthma treatment steps, per copy of Arg16 allele (table 4.2.7).

The Glu27Gln genotype did not show any increase in the risk of requirement for the short courses of oral steroids due to asthma exacerbations in this population (Table 4.2.8). The Glu27Gln genotype did not show any increase in the risk of school absences due to asthma exacerbations in this population (Table 4.2.9).

Table 4.2.10 shows the overall effect of the Glu27Gln genotype on the risk of increase in hospital admissions due to asthma exacerbations in this population, conditioned on the Arg16Gly genotype. Table 4.2.11 shows the overall effect of the Glu27Gln genotype on the risk of increase in overall exacerbations of asthma in this population, conditioned on the Arg16Gly genotype.

The data in table 4.2.12 demonstrates an increased prevalence of exacerbations in individuals per copy of the Arg16 allele in the total population regardless of medication: (OR 1.30, 95% CI=1.09-1.55; p=0.003). As the co-dominant model provided the best fit of the data based on the highest likelihood ratio obtained for this model, this model was used for the remainder of the analysis.

The Arg16 variant did not appear to be associated with general asthma severity as there was no difference in the Arg16 allele frequency across the different treatment steps)(table 4.2.12) (chi-square test 8 d.f. = 5.8, p=0.666).

In this present study which has been extended to 1182 participants, I observed a significant increase in exacerbation risk per copy of the Arg16 allele in participants on treatment step 2 (regular inhaled corticosteroids and inhaled salbutamol on demand) (Gly/Gly 97/271

(36%), Arg/Gly 112/293 (38%), Arg/Arg 47/95 (49%); OR= 1.32; 95%CI=1.04-1.67); *p*=0.02) (table 4.2.12).

I also observed an increase in exacerbation risk per copy of the Arg16 allele on step 3 (regular inhaled steroids, long-acting β_2-agonist salmeterol and inhaled salbutamol on demand) (Gly/Gly 25/63 (40%), Arg/Gly 34/65 (52%), Arg/Arg 19/29 (65%); OR= 1.74 (95%CI=1.09-2.80); *p*= 0.02) (table 4.2.12). This effect was not observed in participants with asthma at steps 0, 1 and 4.

In comparison, this effect was not observed in the Glu27Gln variations (table 4.2.13). Table 4.2.14 shows the association of homozygous Arg16 status with asthma exacerbations in patients on regular salmeterol (combined steps 3 and 4) and those not on regular salmeterol (combined steps 0, 1, and 2). I observed an increased risk of exacerbation in both the groups (combined steps 0, 1 and 2: OR= 1.23, 95% CI= 0.99-1.52, *p*=0.05; and combined steps 3 and 4: OR= 1.51, 95%CI=1.05-2.17, *p*=0.02). Participants in treatment steps 2, 3 and 4 showed greater use of inhaled short-acting β_2-agonists according to need in comparison to participants in treatment steps 0 and 1 (odds ratio for daily or greater use 3.85; 95%CI= 2.86-5.18, *p*<0.001).The Arg16Arg variant was associated with a greater frequency of asthma exacerbations in children with persistent and moderate to severe asthma with more frequent use of short-acting β_2-agonists (i.e. treatment steps 2, 3 and 4 combined) in comparison to those with mild asthma (treatment steps 0 and 1) where short-acting β_2-agonist use is less frequent.

This data suggested that exposure to β_2-agonist *per se* rather than whether the β_2-agonist was short or long acting, was associated with increase in the risk of exacerbations in children carrying the Arg16 allele. I therefore studied the association of the Arg16Gly genotype for asthma exacerbations in individuals on infrequent versus daily exposure to salbutamol and/or salmeterol (table 4.2.15). In asthmatic participants taking any inhaled β_2-agonist less than once a day, there was no effect of Arg16 allele copy on the risk of exacerbations. In participants using any inhaled β_2-agonist at least once daily (as required salbutamol taken at least once daily and/or regular inhaled salmeterol) there is an increased risk of asthma-related exacerbations (OR=1.64 (95% CI 1.22-2.20; p=0.001) per copy of Arg16 allele. The interaction term between genotype and daily use of any inhaled β agonists (as required salbutamol and/or salmeterol) was significant (p=0.022) (table 4.2.11). In the group exposed to β_2-agonists daily, the co-dominant model of risk was clearly observed with the Arg16Gly heterozygotes having an odds ratio for exacerbations of 1.63 (95%CI= 1.02-2.60, p=0.04) and the Arg16Arg homozygotes having an odds ratio of 2.70 (95%CI= 1.46-4.99, p=0.002) when compared to the individuals with the common Gly16Gly genotype.

The Arg16 variant had no significant effects on individual measures of pulmonary function (FEV_1, FVC and PEFR). FEV_1/FVC forced expiratory ratio was significantly reduced in participants with Arg16Arg genetic status with moderate to severe asthma compared to mild asthma (0.84 versus 0.88; p=0.002) (figure 4.2.1) but not in those with the Arg/Gly or Gly/Gly genotypes with moderate to severe asthma compared to mild asthma (0.89 versus 0.90; p>0.05).

Table: 4.2.1: Demographics of participants for the association study between the β_2 agonist genotype variations and asthma exacerbations (n= 1182)

Mean (SD) age (years)	10.3 (4.0) (<18=1137; ≥18= 45)
Sex (Male/Female)	703/479
Mean (SD) body mass index	19.1 (4.5)
Mean (SD) PEFR (% of mean predicted) n=915	87.6 (17.8)
Mean (SD) FEV_1 (% of mean predicted) n=915	95.8 (15.5)
Mean (SD) FVC (% of mean predicted) n=915	92.2 (14.4)
Mean (SD)FEV_1/FVC (% of mean predicted) n=915	102 (0.97)
Atopic eczema (yes/no); n=1182	607/575 (51.4%)
Exposure to smoke (yes/no); n=1182	401/744 (35%)
Arg16Gly genotype distribution; n=1182	Gly16Gly 483 (40.8%), Arg16Gly 518 (43.8%), Arg16Arg 181(15.3%)

KEY: SD: standard deviation; *PEFR*: Peak expiratory flow rate; FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity

Table 4.2.2: Medication use and exacerbations of asthma (n= 1182)

Modified British Thoracic Society (BTS) step of asthma treatment*	0= 39(3.3%); 1= 204 (17.3%); 2= 660 (56%); 3= 157 (13.3%); 4= 117 (10%)
Mean dose of inhaled corticosteroids (Beclomethasone diproprionate equivalent)	432.3 microgram (SEM 12.6)
Number of children using more than once a week 'on demand' inhaled short-acting beta agonists	1044 (79%)
Inhaled bronchodilator use†	0= 137(11.6%); 1= 827(70%); 2= 186(15.7%); 3= 31(2.6%)
School absences (yes/no)over previous 6 months; n=1182	382/ 800 (32.3%)
Courses of oral steroids (yes/no)over previous 6 months; n=1182	263/ 919 (22.3%)
Hospital admissions (yes/no)over previous 6 months; n=1182	157/ 1025 (13.3%)
Overall asthma exacerbations ‡ (yes/no)over previous 6 months; n=1182	456/ 726 (38.6%)

KEY:*** Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand; †Inhaled bronchodilator use: 0= none, 1= occasional (more than once a week and less than daily use), 2= daily (200 mcg/ day required for symptom control), 3= excessive use (use of more than one dose of 200 micrograms/ day for symptom control); ‡Defined as any one of the following in previous 6 months: school absences, courses of oral steroids, or hospital admissions

Table: 4.2.3: Genotype distributions for codons 16 and 27 (n= 1182)

Polymorphic variations	**No. of individuals**
Gly16Gly Glu27Glu	232
Gly16Gly Glu27Gln	197
Gly16Gly Gln27Gln	45
Arg16Gly Glu27Glu	3
Arg16Gly Glu27Gln	372
Arg16Gly Gln27Gln	132
Arg16Arg Glu27Glu	0
Arg16Arg Glu27Gln	0
Arg16Arg Gln27Gln	175
Undetermined at position 27	26

Table: 4.2.4: Overall effect of Arg16Gly genotype on oral steroid intake due to asthma exacerbations, in children and young adults with asthma regardless of treatment

	Genotype					
	Gly16Gly	**Arg16Gly**	**Arg16Arg**	**Total**	**OR (95% CI)**	***p* value**
No	383	404	131	918	1.27 (1.04-1.56)	0.02
Yes	99	113	50	262		
Total	482	517	181	1180		

KEY: OR: Odds ratio; CI: Confidence interval
p values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke. Odds ratio is per copy of the Arg16 allele (additive model).

Table: 4.2.5: Overall effect of Arg16Gly genotype on school absences due to asthma exacerbations in children and young adults with asthma regardless of treatment

	Genotype					
	Gly16Gly	**Arg16Gly**	**Arg16Arg**	**Total**	**OR (95% CI)**	***p* Value**
No	338	355	107	800	1.29 (1.07-1.54)	0.007
Yes	144	162	74	380		
Total	482	517	181	1180		

KEY: OR: Odds ratio; CI: Confidence interval
p values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke. Odds ratio is per copy of the Arg16 allele (additive model).

Table: 4.2.6: Overall effect of Arg16Gly genotype on hospital admissions due to asthma exacerbations in children and young adults with asthma regardless of treatment

	Genotype					
	Gly16Gly	**Arg16Gly**	**Arg16Arg**	**Total**	**OR (95% CI)**	***p* Value**
No	417	452	154	1023	1.04 (0.82-1.34)	0.73
Yes	65	65	27	157		
Total	482	517	181	1180		

KEY: OR: Odds ratio; CI: Confidence interval
p values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke. Odds ratio is per copy of the Arg16 allele (additive model).

Table: 4.2.7: Overall effect of Arg16Gly genotype on overall asthma exacerbations, in children and young adults with asthma regardless of treatment

	Genotype					
	Gly16Gly	**Arg16Gly**	**Arg16Arg**	**Total**	**OR (95% CI)**	***p* Value**
No	310	321	95	726	1.30 (1.09-1.55)	0.003
Yes	172	196	86	454		
Total	482	517	181	1180		

KEY: OR: Odds ratio; CI: Confidence interval
p values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke. Odds ratio is per copy of the Arg16 allele (additive model).

Table 4.2.8: Overall effect of Glu27Gln genotype on oral steroid intake in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype

	Genotype					
	Glu27Glu	**Glu27Gln**	**Gln27Gln**	**Total**	**OR (*p* value) for Glu27Gln**	**OR (*p* value) for Arg16Gly**
No	192	264	442	898	1.11 (0.82-1.48)	1.21 (0.91-1.61)
Yes	43	88	127	258	*p* =0.50	*p*=0.19
Total	235	352	569	1156		

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per genotypic step

Table 4.2.9: Overall effect of Glu27Gln genotype on school absences due to asthma exacerbations, in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype

	Genotype					
	Glu27Glu	**Glu27Gln**	**Gln27Gln**	**Total**	**OR (*p* value) for Glu27Gln**	**OR (*p* value) for Arg16Gly**
No	167	224	393	784	1.08 (0.83-1.39) *p*=0.57	1.24 (0.96-1.59) *p*=0.10
Yes	68	128	176	372		
Total	235	352	569	1156		

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per genotypic step

Table 4.2.10: Overall effect of Glu27Gln genotype on hospital admissions due to asthma exacerbations, in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype

	Genotype					
	Glu27Glu	**Glu27Gln**	**Gln27Gln**	**Total**	**OR (*p* value) for Glu27Gln**	**OR (*p* value) for Arg16Gly**
No	207	303	492	1002	1.18 (0.83-1.66) ***p*** =0.35	0.95 (0.68-1.34) ***p*** =0.79
Yes	28	49	77	154		
Total	235	352	569	1156		

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per genotypic step

Table 4.2.11: Overall effect of Glu27Gln genotype on overall asthma exacerbations, in children and young adults with asthma across all steps of asthma management conditioned on Arg16Gly genotype

	Genotype					
	Glu27Glu	**Glu27Gln**	**Gln27Gln**	**Total**	**OR (*p* value) for Glu27Gln**	**OR (*p* value) for Arg16Gly**
No	153	200	357	710	1.06(0.82-1.36) *p* =0.65	1.27 (0.99-1.63) *p*=0.06
Yes	82	152	212	446		
Total	235	352	569	1156		

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per genotypic step

Table: 4.2.12: Association of asthma exacerbations and Arg16Gly genotype variations according to the steps of management of asthma (n=1182)

Asthma treatment steps	Genotype	Exacerbations over previous 6 months			OR (95%CI) *p* value
		No	Yes	Total	
Step 0	Gly16Gly	11	2	13	0.62 (0.09-4.22) *p*=0.63
	Gly16Arg	19	1	20	
	Arg16Arg	5	1	6	
	Total	35	4	39	
Step 1	Gly16Gly	70	19	89	1.02 (0.57-1.83) *p*=0.93
	Gly16Arg	71	17	88	
	Arg16Arg	23	4	27	
	Total	164	40	204	
Step 2	Gly16Gly	174	97	271	1.32 (1.04-1.67) *p*=0.02
	Gly16Arg	181	112	293	
	Arg16Arg	48	47	95	
	Total	403	256	659	
Step 3	Gly16Gly	38	25	63	1.74 (1.09-2.80) *p*=0.02
	Gly16Arg	31	34	65	
	Arg16Arg	10	19	29	
	Total	79	78	157	
Step 4	Gly16Gly	16	26	42	1.41 (0.77-2.55) *p*=0.26
	Gly16Arg	19	32	51	
	Arg16Arg	8	15	23	
	Total	43	73	116	

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model- that is, a gene/dosage effect for the Arg16Arg variant. Odds ratios are per copy of the Arg16 allele. There was no difference in the distribution of the Arg16 variant between the treatment steps (chi-square test 8 d.f. = 5.8, *p*=0.666)

Table 4.2.13: Association of asthma exacerbations and Glu27Gln genotype variations according to the steps of management of asthma, conditioned on Arg16Gly genotype

Asthma treatment steps	Genotype	Exacerbations over previous 6 months			OR (P value)
		No	Yes	Total	
Step 0	Glu27Glu	5	1	6	0.17 (0.11)
	Glu27Gln	16	2	18	
	Gln27Gln	12	1	13	
	Total	33	4	37	
Step 1	Glu27Glu	32	11	43	1.50 (0.33)
	Glu27Gln	90	17	107	
	Gln27Gln	38	11	49	
	Total	160	39	199	
Step 2	Glu27Glu	90	48	138	0.99 (0.95)
	Glu27Gln	199	125	324	
	Gln27Gln	107	80	187	
	Total	396	253	649	
Step 3	Glu27Glu	20	8	28	1.30(0.41)
	Glu27Gln	32	32	64	
	Gln27Gln	25	34	59	
	Total	77	74	151	
Step 4	Glu27Glu	6	11	17	0.74 (0.48)
	Glu27Gln	19	36	55	
	Gln27Gln	17	26	43	
	Total	42	76	115	

KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per genotypic step

Table 4.2.14: Effect of Arg16Gly genotype status on the proportion of patients developing asthma exacerbations according to salmeterol treatment status.

Treatment	**Genotype**	**Total exacerbations**			**OR (95% CI) (*p* values)**
		No	**Yes**	**Total**	
No Salmeterol	Gly16Gly	255	118	373	1.23 (0.99-1.52) *p*=0.05
	Gly16Arg	271	130	401	
	Arg16Arg	76	52	128	
	Total	602	300	902	
Salmeterol treated.	Gly16Gly	54	51	105	1.51 (1.06-2.17) *p*=0.02
	Gly16Arg	50	66	116	
	Arg16Arg	18	34	52	
	Total	122	151	273	

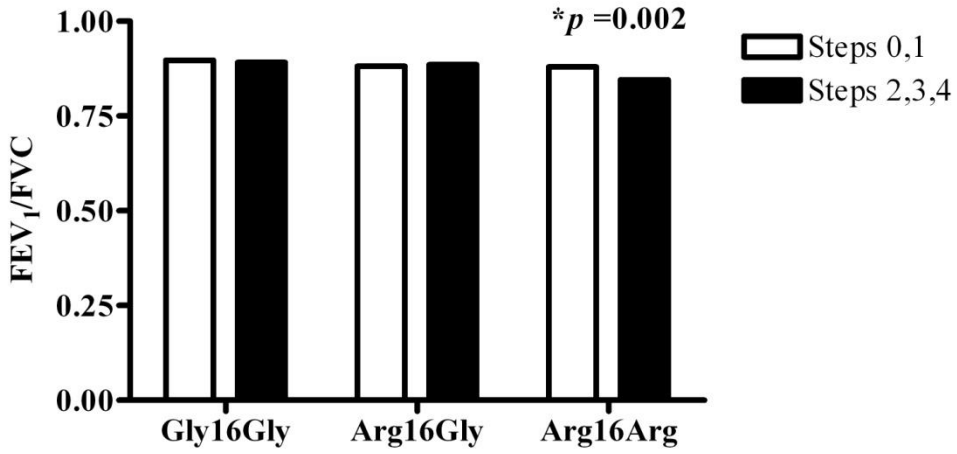
KEY: *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. Odds ratios are per copy of the Arg16 allele.

Table 4.2.15: Association and drug-genotype interaction of Arg16Gly genotype with asthma exacerbations in children with, and without, daily exposure to β_2 agonists.

Exacerbations		**NO**	**YES**	**Total**	**OR (95% CI)** ***p*** **value**
Salbutamol/salmeterol usage less than once per day.	Gly16Gly	228	100	328	1.08 (0.85-1.36) *p* =0.525*
	Arg16Gly	241	98	339	
	Arg16Arg	69	37	106	1.27 (0.77-2.08) *p* =0.353**
	Total	538	235	773	
Exacerbations		**NO**	**YES**	**Total**	
Daily use of any β agonist (as required salbutamol and/or salmeterol)	Gly16Gly	81	69	150	1.64 (1.22-2.20) *p* =0.001*
	Arg16Gly	80	97	177	
	Arg16Arg	25	49	74	2.68 (1.46-4.94) *p* =0.002**
	Total	186	215	401	
	Test for interaction between genotype and daily use of any β agonist on exacerbations.				*p* =0.022* *p* =0.049**

KEY**:** *p* values were calculated by binary logistic regression corrected for age, sex, perennial rhinitis, smoking and exposure to tobacco smoke, using a co-dominant model. *Odds ratios (OR, 95%CI) and *p* values are shown for the co-dominant model (Odds ratio per copy of the Arg16 allele). **Odds ratios (OR, 95%CI) and *p* values are shown for the comparison of Arg16 homozygotes versus the Gly16 homozygotes.

Figure 4.2.1: Association of airway obstruction measured as FEV_1/FVC and β_2 adrenergic receptor gene variations in children with mild (treatment steps 0 and 1) and moderate to severe (treatment steps 2, 3, 4) asthma



KEY: FEV_1: Forced expiratory value at 1 second; FVC: Forced vital capacity

Chapter 3

Effects Of Filaggrin Null Mutations In Asthma Exacerbations

The demographic characteristics of the children who participated in the study to explore the association between *FLG* null mutations and asthma exacerbations are described in table 4.3.1. Table 4.3.2 describes the management of asthma as per the modified BTS guideline steps, inhaled bronchodilator use, filaggrin genotypes and measures of asthma exacerbations in the participants. Figure 4.3.1 describes the proportion of the study population with *FLG* null allele on the treatment steps of asthma management.

Figure 4.3.2 demonstrates frequencies of individuals with *FLG* null mutations with and without asthma exacerbations over the previous 6 months. Amongst the participants without an asthma exacerbation, only 11.2% had an *FLG* null mutation. 16.8% of participants with asthma exacerbation had an *FLG* null mutation. The allele frequencies of the *FLG* mutations R501X and 2282del4 in the children with asthma were increased relative to the Tayside population.

Table 4.3.3 describes the association between the filaggrin null mutations and asthma exacerbations. Asthma exacerbations were found to be significantly increased in children with *FLG* mutation R501X and the combined genotype. The contingency analysis (Table 4.3.3) shows that the heterozygous and homozygous genotypes for the R501X mutation, and the combined genotype, were associated with higher risk for exacerbations of asthma.

This is significant for the R501X mutation (p= 0.009) and the combined genotype (p= 0.021; Table: 4.3.3). 35% (301/859) of *FLG* wild- type participants were prone to exacerbations, a significantly greater proportion 51% (35/68) of *FLG* null allele carriers with asthma suffered from exacerbation of their asthma. Hence there was a 1.97- fold greater risk (95% CI, 1.19- 3.22) of suffering from exacerbation of asthma in *FLG* null allele carriers in comparison to *FLG* wild- type participants with asthma.

Table 4.3.5 shows that there was a significant increased risk in asthma-related school absence in participants carrying R501X mutation (p= 0.041). 30.0% (261/862) and 19.5% (173/ 887) of *FLG* wild type participants were absent from school or required oral steroids due to worsening of their asthma. This compares with 42.6% (29/68) and 31.4% (22/70) of *FLG* null allele carriers experiencing school absences or requiring a course of oral steroids over the previous 6 months. There was a 1.71-fold risk (95% CI, 1.04- 2.83) of school absences due to asthma and a 1.89- fold risk (95% CI, 1.11-3.21) of requiring oral steroids to treat exacerbations in this population.

No such association was observed in relation to hospital admission due to asthma exacerbations over the previous 6 months of collection of the data (table 4.3.6).

Figure 4.3.3 describes the proportion of the total study population with asthma exacerbations, irrespective of their genotype, in the previous 6 months across the treatment steps of asthma management according to the modified BTS guidelines. The participants on

step 3 of asthma management constituted the maximum proportion (48%) of children with asthma exacerbations over the previous 6 months. This was followed by participants on step 2 (37%).

On further analysis, exacerbations of asthma were found to be significantly increased (OR 1.83, 95% CI, 1.013- 3.29; p= 0.045) in individuals with the *FLG* null alleles compared to *FLG* wild type only for asthma treatment step 2 (regular inhaled steroids plus inhaled short-acting beta agonists according to need) although a similar trend with greater risk with *FLG* null alleles compared to *FLG* wild type was observed for treatment steps 3 and 4 (participants on regular inhaled long-acting beta agonists with or without montelukast, in addition to regular inhaled steroids and inhaled short-acting β-agonists according to need) .

Table: 4.3.1: Demographic characteristics of participants with asthma for the study of association between asthma exacerbations and filaggrin mutations (n=1135)

Age	Range, 3-22 (mean, 10.3; SD, 5.1); (<18=1091; ≥18= 44)
Sex (males: females)	671 (59.1%): 464 (40.9%)
With eczema: without eczema (n=1125)	580:544
Family history of asthma and eczema	
Paternal asthma (yes/ no)	219/ 901 (19.6%)
Paternal eczema (yes/ no)	82/ 1037 (7.3%))
Maternal asthma (yes/ no)	269/ 850 (24.0%))
Maternal eczema (yes/ no)	162/ 958 (14.5%)
Mean percent predicted FEV_1 (SD) (n=863)	95.8 (15.6)
Mean percent predicted FVC (SD) (n=862)	92.2 (14.5)
Mean FEV_1/ FVC (SD) (n=880)	89.3 (14.8)

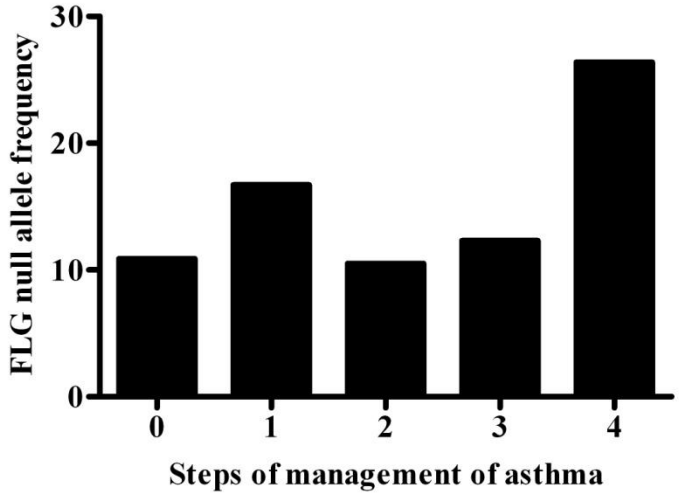
KEY: SD: standard deviation; PEFR: Peak expiratory flow rate; FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity

Table 4.3.2: Medication use, genotypes and asthma exacerbations

Modified BTS asthma treatment steps †(n=1116)	Step 0=69; Step 1=189; Step 2= 610; Step 3=149; Step 4=99
Inhaled bronchodilator use (n=1111)	
0= None	144
1= Occasional	763
2= Daily	178
3= Excessive	26
R501X AA: Aa/ aa (%)	895(92.6%): 72 (7.4%)
2282del4 AA: Aa/ aa (%)	856 (94.3%): 52 (5.7%)
Combined genotype AA: Aa/ aa (%)	774 (86.7%): 119 (13.3%)
School absences (yes/ no) over previous 6 Months	339/ 753 (31%)
Courses of oral steroids (yes/ no) over previous 6 months	228/ 895 (20.3%)
Hospital admissions (yes/ no) over previous 6 months due to exacerbations	125/ 998 (11.1%)
Overall asthma exacerbations *(yes/ no) over previous 6 months	394/ 694 (36.2%)

KEY: †Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand; aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation; *Defined as any one of the following in previous 6 months: school absences, courses of oral steroids, or hospital admissions

Figure 4.3.1: The proportion of participants with *FLG* null allele frequency across the treatment steps of the management of asthma



KEY: Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand

Figure 4.3.2: The frequencies of individuals with *FLG* null mutations with and without asthma exacerbations over the previous 6 months.

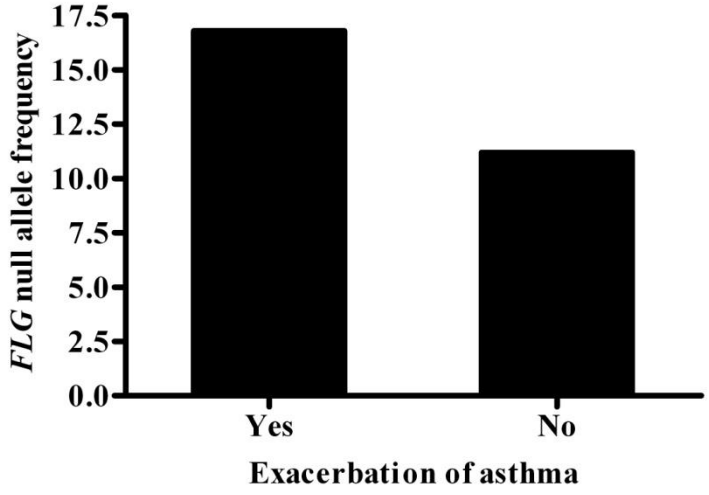


Table 4.3.3: Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus exacerbations of asthma.

		Yes	No	Total	*p*-value (one tailed)	*p* Value (two tailed)	Odds ratio (95% CI)
R501X	AA	301	558	859	0.006	0.009	1.97 (1.19-3.23)
	Aa/aa	35	33	68			
	Total	336	591	927			
2282del4	AA	295	526	821	0.438	0.764	1.09 (0.61- 1.97)
	Aa/aa	19	31	50			
	Total	314	557	871			
Combined genotype	AA	257	486	743	0.013	0.021	1.61 (1.08-2.40)
	Aa/aa	52	61	113			
	Total	309	547	856			

KEY*:* aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation; CI: Confidence interval; Exacerbation was measured as school absence and/or asthma related hospital admissions and/or use of short courses of oral steroids.

Table: 4.3.4: Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus short courses of oral steroids for treatment of asthma exacerbations in the previous 6 months

		Yes	No	Total	*p*-value (one tailed)	*p*-value (two tailed)	Odds ratio (95% CI)
R501X	AA	173	714	887	0.016	0.021	1.89 (1.11-3.22)
	Aa/aa	22	48	70			
	Total	195	762	957			
2282del4	AA	167	680	847	0.203	0.366	1.39 (0.73- 2.67)
	Aa/aa	13	38	51			
	Total	180	718	898			
Combined genotype	AA	145	622	767	0.014	0.025	1.71 (1.09-2.65)
	Aa/aa	33	83	116			
	Total	178	705	883			

KEY: aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation; CI: Confidence interval

Table: 4.3.5: Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus asthma-related school absences in the previous 6months

		Yes	No	Total	*p*-value (one tailed)	*p*-value (two tailed)	Odds ratio (95% CI)
R501X	AA	261	601	862	0.026	0.041	1.71 (1.04-2.83)
	Aa/aa	29	39	68			
	Total	290	640	930			
2282del4	AA	259	565	824	0.173	0.345	0.69 (0.35-1.34)
	Aa/aa	12	38	50			
	Total	27	603	874			
Combined genotype	AA	228	518	746	0.229	0.445	1.19 (0.79-1.82)
	Aa/aa	39	74	113			
	Total	267	592	859			

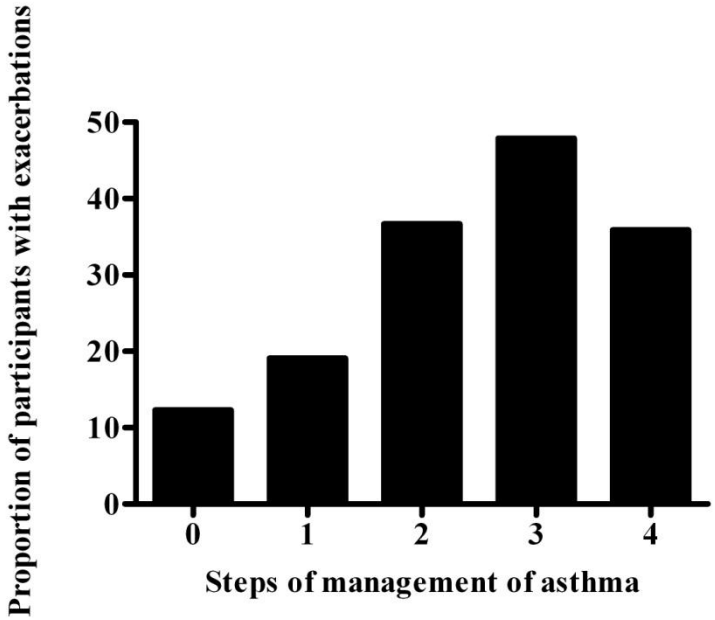
KEY: aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation; CI: Confidence interval

Table: 4.3.6: Contingency table for the filaggrin genotype (co-dominant and mutant variants) versus asthma-related hospital admissions in the previous 6months

		Yes	No	Total	*p*-value (one tailed)	*p*-value (two tailed)	Odds ratio (95% CI)
R501X	AA	94	792	886	0.337	0.548	1.24 (0.59-2.58)
	Aa/aa	9	61	70			
	Total	103	853	956			
2282del4	AA	89	757	846	0.374	0.812	0.72 (0.25-2.06)
	Aa/aa	4	47	51			
	Total	93	804	897			
Combined genotype	AA	80	686	766	0.453	0.748	1.08 (0.58-2.01)
	Aa/aa	13	103	116			
	Total	93	789	882			

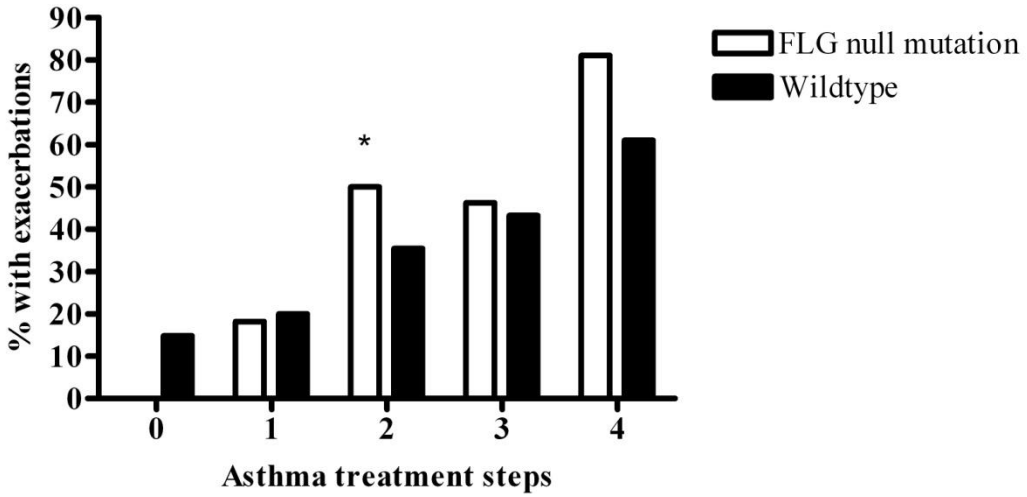
KEY: aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation; CI: Confidence interval

Figure 4.3.3: The proportion of participants with exacerbations of asthma across the steps of management of asthma



KEY: Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand

Figure 4.3.4: Comparison of asthma exacerbations between children and young adults with and without *FLG* null alleles across the treatment steps of asthma



KEY: **p*=0.045; Step 0 – no use of inhaled salbutamol on demand within the past month; Step 1 - inhaled salbutamol on demand; Step 2 - regular inhaled steroids plus inhaled salbutamol on demand; Step 3 - regular inhaled salmeterol plus inhaled steroids with inhaled salbutamol on demand; step 4 - regular inhaled salmeterol plus inhaled steroids plus oral montelukast and/or other add-on medications with inhaled salbutamol on demand

Chapter 4

Effects of filaggrin defects on epithelial permeability

The demographic characteristics of the participants participated in the pilot study to determine the effects of filaggrin defects on the epithelial permeability in skin and gut are described in table 4.4.1. 10 children with asthma with or without eczema were recruited as well as 14 children as control population. This was not a blinded study and the control group was not pre-genotyped.

Figure 4.4.1 shows the individual measurements of the transepidermal water loss in g/hm^2 of the asthmatic children with filaggrin mutation (median ±MAD= 11.3 ± 1.75g/hm^2, n=10) and the control group (median ±MAD= 7.45±1.38 g/hm^2, n=14). Transepidermal water loss in the asthmatic children heterozygous for the filaggrin null alleles was significantly increased (p=0.02, Mann Whitney rank sum test). One child in this group had very high measurement (33g/hm^2) and therefore the median was recalculated omitting this outlier (10.8 ± 1.8g/hm^2, n=9). The transepidermal water loss of this group was still significantly higher than that of the control group (p=0.044, Mann Whitney rank sum test).

The percentages of ingested lactulose recovered in the urine of the control group (median±MAD= 0.18±0.006%, n=8) were not different from those of the children heterozygous for the filaggrin null alleles (median±MAD=0.20±0.006%, n=8, p=not significant Mann Whitney rank sum test). The percentages of ingested mannitol recovered

in the urine were (11.9±7.8%, n=8) in the control group, not different from those of the children heterozygous for the filaggrin null alleles (16.3±7.5%, n=8, p=not significant Mann Whitney rank sum test). The lactulose/ mannitol ratio has been plotted for each individual in figure 4.4.2.

Lactulose: mannitol ratios of the control group (0.013±0.010) were also not different from the ratios of the children heterozygous for the filaggrin null alleles (0.014±0.007, p=not significant, Mann Whitney rank sum test).

Table 4.4.1: Characteristics of controls and participants with filaggrin mutations for the epithelial permeability pilot study

Characteristic	**Asthma patients with *FLG* mutations (n=10)**	**Control group (n=14)**
Eczema	7 (70%)	0
Age ± SD	10.9 ±3.2	9.9 ± 2.9
Sex (males: females)	6: 4	8:6
R501X AA: Aa/ aa	1:9	Not applicable
2282del4 AA: Aa/ aa	9:1	Not applicable
Combined genotype AA: Aa/ aa	1: 9	Not applicable
Paternal asthma (yes/ no)	3/7(30%)	None
Paternal eczema (yes/ no)	2/ 8 (10%)	None
Maternal asthma (yes/ no)	3/7 (30%)	None
Maternal eczema (yes/ no)	3/7 (30%)	None

KEY: aa: Homozygous R501X or 2282type or compound heterozygous genotype; Aa: Heterozygous genotype for either R501X or 2282del4; AA: Wild-type FLG genotype for R501X and 2282del4 mutation

Figure 4.4.1: Transepidermal water loss in the children heterozygous for the *FLG* null allele with asthma and the control group

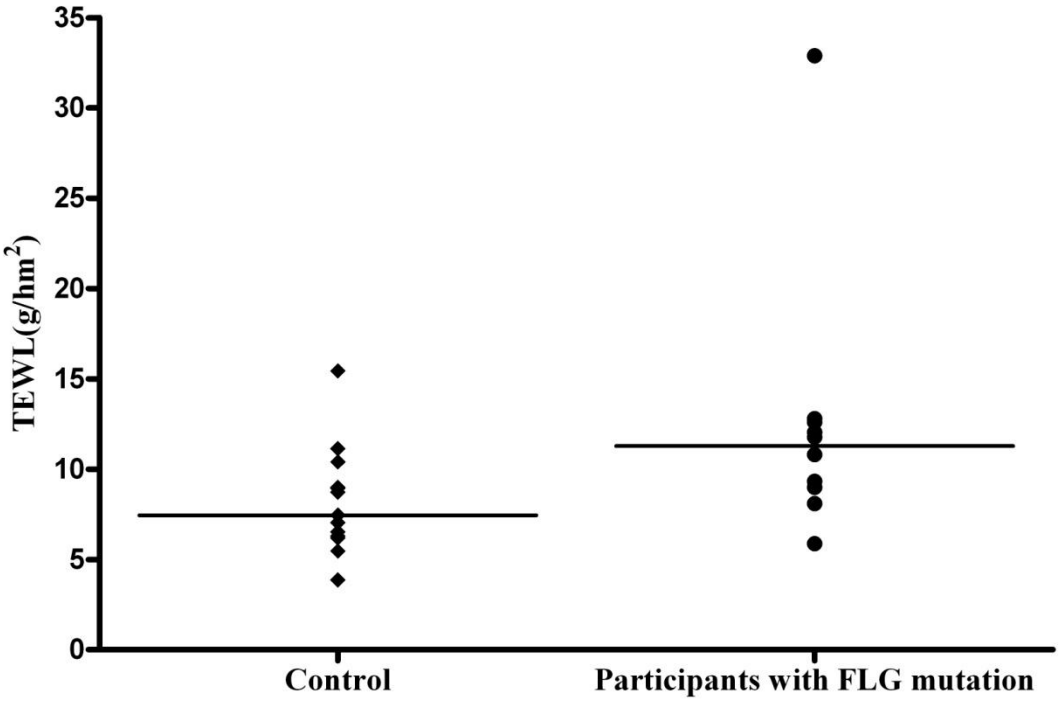
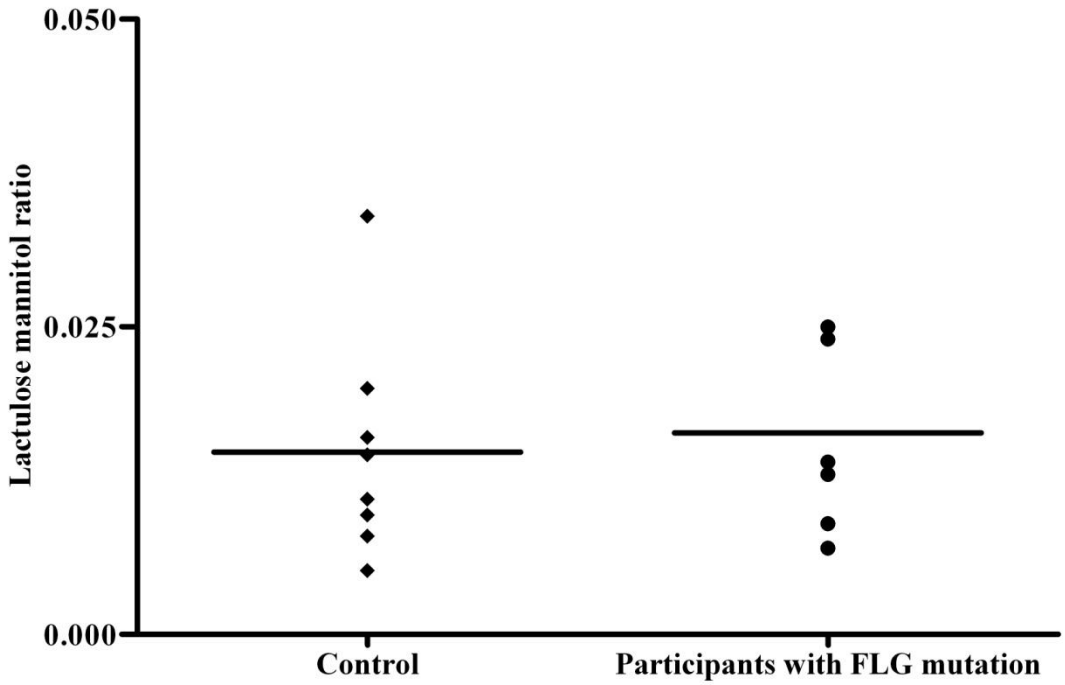


Figure 4.4.2: Excreted lactulose/mannitol ratio in the children heterozygous for the *FLG* null allele with asthma and the control group



SECTION V

DISCUSSION

Chapter 1

BREATHE Is A Large Cross-Sectional Study In Children's Asthma With Genotype And Phenotype Data

For the first time, a large cross-sectional study has been conducted in a population of children and young adults with asthma in primary and secondary care in the UK, in terms of relevant history, medication use and exacerbations. The study of genetic variations in relation to response to medication in asthma will allow us to gain key information regarding which patients are best suited for a particular therapeutic agent and which patients may be at risk for serious potentially life-threatening complications from standard treatment regimens.

Characterizing persistent users of asthma medication is important to understand prescribing of asthma medication in children[118]. However, studies performed to assess trends in paediatric asthma drug prescribing in the UK, have shown that the increase in the number of combination inhalers prescribed is not consistent with the guideline recommendations[32] and off-label prescribing for children with asthma in primary care was found to be

associated with worse levels of self-reported asthma control[34]. Children and young adults with asthma also receive treatment not in accord with current licences and evidence-based recommendations and, as such, may be at risk of adverse outcomes[33]. A previous study highlights the over-use of high-dose inhaled corticosteroids, the under-use and inappropriate use of add- on therapy, and the use of very high and potentially dangerous doses of inhaled corticosteroids in a minority of children[33].

Marked variability in severity of symptoms, natural history, risks of adverse outcomes, pathologic characteristics, and response to therapy have been observed in asthma[5]. A striking absence of correlation is observed among the pathologic, physiologic, and clinical manifestations of asthma in adults[5]. Lung function tends to correlate poorly with clinical outcomes, and there is only modest correlation between clinical outcomes. Understanding the relationship between these factors may also be complicated by the variability of outcomes and the lack of correlation between them.

The BREATHE database of children and young adults with asthma contains information on patient genotype, environmental factors contributing to asthma, and clinical asthma severity. Children's asthma has distinctive features and does not necessarily lead to asthma in adult life. Several novel observations on the gene-environmental interactions associated with childhood asthma, obtained from the BREATHE study have been published[22-24,103-104]. The results of these preliminary studies in children have allowed me to proceed to hypothesis-based research that will explore the relationships between phenotypic asthma and specific genotypes. A recent editorial in Nature Genetics expressed concern about

quality of many published case/ control studies that had undermined public confidence when results were first publicised, and then rapidly followed by non-replication or refutation[119]. It suggested the optimal condition for association studies including large sample sizes, small *p* values, and plausible associations that make biological sense. Furthermore, findings must be supported by independent replication, with associations combining family- based and population- based analysis, with an odds ratio and/ or attributable risk that is high. We have been able to replicate gene-environmental associations in relation to asthma, in a paediatric population through several studies originating from the BREATHE database[21, 104].

The database captures a window in the participants' life (six months) and they may have had changes to treatment just prior to the data acquisition (particularly if they have only recently attended the clinic). This might account for some of the findings in relation to children and young adults with asthma on treatment step 4. This is a major limitation of the study. However, for simplicity and better parental recall of events, the history was taken and limited only for the previous six months.

The study is now being extended to Sussex with the aim of creating a larger database across the UK. The aim is to recruit more participants over the next few years and study similar genetic variations in relation to clinical outcomes. The Sussex database would be subsequently merged with the Scottish database giving us information from a bigger population. The diversity in ethnic, cultural and environmental background of the

population might help in the exploration of several other associations in asthma phenotypic variation and specific genotypes.

Chapter 2

Adrenergic β_2-receptor Genotype Predisposes To Exacerbations In Steroid-Treated Asthmatics On Frequent Salbutamol Or Salmeterol

My study (tables 4.2.1-4.2.15; figure 4.2.1) shows for the first time that there is an increase in risk of exacerbations per copy of Arg16 allele in children and young adults with asthma on frequent (once daily or more) as required doses of inhaled salbutamol (table 4.2.15). This effect is not observed in participants with asthma who are not exposed to β_2-agonist on a daily basis. The study also extends the previous findings of an increase in the risk of exacerbations per copy of Arg16 allele in children and young adults with asthma on the regular long-acting β_2-agonist salmeterol. The number of subjects on salmeterol in the previous paper was 164 and the group studied in this thesis comprises 273 individuals on salmeterol.

I have only investigated two of the SNPs in the *ADRB2* gene. Other haplotypes of the *ADRB2* gene have been described[64, 120]. However, a closer study of the haplotype data show that only 3 of the haplotypes are found in relevant numbers in the Caucasian population and these are completely tagged by codon 16 and codon 27[64,120]. It is of particular note that the promoter gene variations are in complete linkage disequilibrium with the Arg16 variant.

This linkage disequilibrium is exclusively relevant in the Caucasian population. Therefore in practical terms, in a study such as ours where the analysis exclusively involves Caucasians, it is only possible to study the association of the 16/27 diplotypic variation with clinical outcomes. However, the possibility remains that associations with the Arg16 variant may only be due to an uncharacterised and tightly linked causal variant.

Both retrospective and prospective analyses of data from a number of randomized controlled trials have demonstrated the adverse effects of the Arg16 variant on pulmonary function (peak flow rates[60,61,65], FEV_1[60,61]), asthma symptoms[60,65], methacholine responsiveness and exacerbations[61,71,121] and 'reliever' bronchodilator use[60] in adults with asthma on regular short-acting β_2-agonists. Inhaled short-acting β_2-agonists used on an 'on demand' basis constitute one of the most commonly prescribed asthma medications in the world[18]. This method of use results in a very different pattern of administration of short-acting β_2-agonists, as compared to the regular use of this medication, with short-acting β_2-agonists typically being used prior to activity (e.g. football), exposure to cold (e.g. going to school in the early morning), or as frequent doses to afford relief during developing asthma exacerbations.

Importantly, the interaction of Arg16 status with inhaled salbutamol on exacerbation risk is observed in a population who are not taking regular doses of inhaled salbutamol that may be comparable to regular qid administration. 70% of the population on treatment steps 2, 3 and 4 were using less than one daily dose (200 micrograms/ day) of inhaled salbutamol. Only 3% of the population was using inhaled salbutamol several times daily (classified as

‘3’ or ‘excessive’: this represents the use of more than one dose of 200 micrograms / day for the control of symptoms). Against this background of relatively sparse ‘on demand’ inhaled short-acting β_2 agonist administration, I have identified a significant deleterious effect on asthma exacerbations per copy of Arg16 allele (table 4.2.15). The interaction between genotype and daily use of any inhaled β_2-agonists (as required salbutamol and/or salmeterol) had a significant effect on the risk of asthma exacerbations (p=0.022) (table 4.2.15).

I have also identified a significant increase in risk of exacerbations in children and young adults with mild persistent asthma (i.e., receiving inhaled steroids with inhaled short-acting β_2-agonists according to need) per copy of Arg16 allele (table 4.2.12). No similar associations were observed in mild intermittent asthmatics (where inhaled salbutamol alone controls intermittent symptoms or where there has been no requirement for inhaled salbutamol within the past month) (table 4.2.12). Indeed, this increased risk per Arg16 allele copy was also present in step 3 patients who were also taking regular long-acting β_2-agonists (13.3% of the overall population) (table 4.2.12). However, in step 4 patients (receiving montelukast in addition to regular long-acting β_2-agonists, inhaled steroids and salbutamol according to need; 9.9% of the overall population), there was no increase in the risk of exacerbations per Arg16 allele copy; despite a higher exposure to short-acting β_2-agonists (table 4.2.12). There was a significant increased risk of exacerbations in the asthmatics when steps 3 and 4 were combined together (OR= 1.51 (95% CI=1.06-2.17), p=0.02) (table 4.2.14). One possible explanation for this apparent disconnect (table 4.2.12) between the genotype associations for steps 3 and 4 could be the concomitant use of

montelukast, or the use of oral steroids (treatment step 3: 34%, treatment step 4:42%) or higher dose of inhaled steroids (treatment step 3: 533 microgram , treatment step 4:561microgram BDP equivalent), the latter protecting against β agonist induced down-regulation and desensitisation via the glucocorticoid response element on the *ADRB2* gene, while the former conferring non steroidal anti-inflammatory protection[122].

Previous observations had shown an increased hazard for exacerbations per copy of Arg16 allele in young asthmatics, with a particular effect of regular inhaled salmeterol on this increase in risk[21]. Now that I have doubled our study size, previous effects remain consistent. However the effect has not become more significant with greater numbers. It is felt, however, that the increased hazard for exacerbations per copy of Arg16 allele in asthmatics on salmeterol, observed in the previous study and subsequently in this extended study, is a true effect. This is because, in the previous study, the children were mostly recruited from the secondary care setting and were on higher steps of asthma management (40% of study population on modified BTS step 3 and above) [18]. When the study was extended, I recruited mainly from the primary care setting, and a smaller proportion of the new recruits are on inhaled salmeterol (in the combined analysis 23% of the study population is on modified BTS step 3 and above). Hence a smaller proportion of the overall participants are on salmeterol in the extended analysis, in comparison to the previous work[21]. This accounts for the observation that while the effect is consistent, it has not become any stronger with the increase of numbers due to a lack of power in step 4.The strategy for recruiting participants from primary care to recruit more patients on inhaled short-acting β_2 agonists on demand and not regular inhaled long-acting β_2 agonists was

guided by the hypothesis that I primarily wished to test for this study, which is that inhaled short acting β_2-agonists interact with the Arg16 genotype to increase exacerbation risk and I wished to specifically recruit a large population of asthmatics on inhaled short-acting β_2-agonists on demand who were not exposed to regular inhaled long-acting β_2 agonists to fulfil this goal.

I have further demonstrated that there is an increase in risk of the individual constituents of the asthma exacerbation score - school absences and requirement for courses of oral steroids – per copy of the Arg16 allele, over a 6-month period of reporting, in addition to the overall effect on asthma exacerbations as has been reported in our previous analysis (tables 4.2.3, 4.2.4, 4.2.6).These individual measures represent different aspects of asthma exacerbations that have different effects on children and young adults in the community. School absences have an impact on the children and young adults' education and work, and oral steroid courses represent the use of primary care whereas hospital admissions represent the impact on secondary care services. Therefore these findings appear to have a greater relevance for the burden of asthma in the community and primary care than hospital settings.

The observation of an adverse effect per copy of the Arg16 allele of 'on demand' short acting β_2-agonists on asthma exacerbation risk raises a further question. As patients on inhaled long-acting β_2-agonists are also on short-acting β_2-agonists, what proportion of the adverse effect of increased risk of exacerbations in the 'real-life' scenario results through the use of long-acting, in comparison to the use of short-acting, β_2-agonists? This question may be resolved through a study that involves the randomisation of asthmatic carrying the

Arg16 allele who are on regular salmeterol to comparator arms of 'reliever' medication, short-acting β_2-agonist versus ipratropium bromide, with risk of asthma exacerbations as the primary outcome measure. Another strategy might be to randomise patients carrying the Arg 16 allele to receive either salmeterol or montelukast as add on to inhaled corticosteroids, with salbutamol reliever in both arms, on the basis that any potential adverse effect of salbutamol could be seen in both randomised arms.

The study participants used inhaled short-acting β_2 agonists as reliever and did not use non β_2 agonists as reliever medication for their asthma. Hence, I cannot provide the data in this study to address the hypothesis that the use of relievers that do not act via the β_2 receptor pathway is not associated with the deleterious effects that have been reported with inhaled β_2 agonist relievers. This is a major limitation of the study. Hence, as these necessary controls are missing, it is too early to draw conclusions in this area of research. Therefore, while these data are of interest, a proper study involving a non β_2 reliever is now required. This could involve either of two strategies. Firstly, if I could identify a population of asthmatics with significant use of non- β_2 relievers, I could compare the interactions of the Arg16 genotype with inhaled β_2 agonist reliever use versus non β_2 agonist reliever use. Alternatively, it could involve a strategy for the prospective randomisation of a population of asthmatics carrying the Arg16 genotype to inhaled β_2 agonist versus non β_2 agonist reliever therapy with an exploration of the hypothesis that there is an increased risk of asthma exacerbations in participants on inhaled β_2 agonists in comparison to participants on non β_2 agonists.

A recent analysis of two separate studies performed on adults (mean age of participants around 40 years for both studies) has failed to demonstrate a role for Arg16 genotype on clinical outcomes including exacerbations, pulmonary function, and asthma control measures in patients receiving inhaled long-acting β_2-agonists[123]. However, the selection criteria for the first study raise questions about its generalisability. In that study, all patients had to demonstrate 12% or more reversibility to inhaled terbutaline and bronchodilator reversibility for the patients had a very high mean value of 24.5%. Selecting such a population has the potential to bias the population towards a group that is disproportionately responsive to all β_2-agonists (i.e. to both short and long acting). Such a population may be less likely to demonstrate the pharmacogenetic effect under study for the analysis. In addition, it has been observed that mean FEV_1 values are much higher (between 90-97% of mean predicted) in asthmatic children attending clinics in Northern Europe[124-126]. A post hoc analysis of a randomized controlled trial on adults with asthma shows that, relative to Gly/Gly patients with asthma, Arg16Arg patients may have an impaired therapeutic response to salmeterol, measured as improvement in lung function, either in the absence or the presence of inhaled corticosteroids[127, 128]. In the previous analysis (432 participants with lung function data) [21], demonstration of an effect of Arg16 homozygous status on airway obstruction was not observed. In the current analysis on over twice the population size (n=879 with FEV_1/FVC values), I have shown that, while FEV_1/FVC is reduced overall in this population (mean 0.88), there is a small but significant reduction in FEV_1/FVC in participants on higher steps of asthma treatment only in the Arg16Arg homozygous population of young asthmatics (0.88 versus 0.84; P=0.002) (figure 4.2.1). It

is doubtful whether such a small difference in forced expiration ratio has any clinical relevance.

In addition, results from studies on adult patients with asthma[123] cannot often be extrapolated to children and teenagers with the disease. Long-term cohort studies have shown that asthma in childhood differs markedly from asthma during adult life[129]. For example, early sensitisation to inhaled allergens constitutes a significant risk factor for asthma until the teenage years, but not for adult asthma[127, 130,131]. Other aspects of asthma, e.g. eosinophil response[130-132] and effects of maternal smoking[133], show major differences between children and adults with asthma. This suggests that the effects of polymorphic variations that are important from the pharmacogenetic perspective could be different between children and adults with asthma. The development of tolerance to the non-bronchodilator (e.g. mast cell-mediated) actions of inhaled β_2-agonists in patients with relatively mild asthma may also interact with these effects[134]. With the vast majority of my participants being aged <15 years (86%) and with the maximum age of participants being 22 years, this study firmly focuses on children with this disease. I feel that the exploration of the role of *ADRB2* genotype primarily in childhood complements the observations of other studies on adults, providing a more complete picture of the effects of this genotypic variation across asthmatics of different age-groups.

Chapter 3

Filaggrin Null Mutations Are Associated With Increased Asthma Exacerbations In Children And Young Adults

Since completion of the data collection for the initial study[22], recruitment of patients with asthma has continued for the Scottish cohort primarily ascertained with asthma to generate statistical power to investigate further the possible roles of filaggrin gene defects on asthma medication use[23] and, subsequently, risk of asthma exacerbations[103]. The data demonstrate that individuals with *FLG* null alleles have a significantly increased risk of exacerbations requiring hospital admissions, courses of oral steroids, or experiencing school absences[103].

On sub-group analysis, the effect of *FLG* mutations on asthma exacerbations is significant only for participants with relatively mild asthma controlled on inhaled steroids, with inhaled salbutamol according to need. There is, however, a trend in the direction of greater morbidity in the presence of *FLG* mutations in participants on higher steps of asthma treatment (i.e. additional inhaled long-acting beta agonists with or without oral montelukast; Figure 4.3.4). This occurs against a background of an overall increasing prevalence of asthma exacerbations with greater asthma medication use (Figure 4.3.3). The overall prevalence of the *FLG* null alleles was higher in participants reporting asthma exacerbations in the previous six months in comparison to those that did not (46.0% versus 34.5%), and this overall difference was significant (p= 0.01; Figure 4.3.2).

The individual contribution to the overall signal (exacerbations) of the 2282del4 allele was lower than that observed for the R501X mutation. In the previous study, a differential penetrance of these two mutations on the requirements for asthma medication was observed[23], and other studies have seen a lower penetrance of the 2282del4 allele in asthma related phenotypes, but not eczema related phenotypes[135]. The mechanism of this is not known, but may be related to an as yet uncharacterised functional difference in individuals with the 2282del4 allele which has the potential to encode filaggrin repeats, which is definitely not the case for the R501X allele, which truncates the protein at the beginning of the first repeat. Interestingly a milder eczema phenotype has been reported for mutations that are much further towards the 3′ end of the gene, although it has been difficult to detect any functional filaggrin in these individuals[73, 84]. Further work is required to delineate possible mechanistic differences in these alleles that may lead to different disease susceptibility. A significantly greater proportion of *FLG* null allele carriers with asthma were on higher BTS treatment steps 3 and 4 [23]. This work reinforces the position that epithelial barrier defects resulting from *FLG* mutations have an major influence on day-to-day aspects of asthma management and control, including overall risk of asthma exacerbations[103], use of oral steroids[103], together with 'as required' doses of inhaled bronchodilators[23] and regular asthma medication needs[23]. *FLG* gene status appears to influence the overall burden of disease in asthmatic children and young adults. An understanding of the possible relationship between filaggrin gene defects and asthma might unfold newer hypotheses that focused primary prevention strategies for asthma may be particularly cost effective and beneficial in specific genotype- stratified populations[27.]

Exacerbations cause the greatest concern to individuals with asthma and can be life-threatening. They also account for the largest proportions of health costs of asthma[136]. Exacerbations of asthma symptoms diminish the quality of life of the patients and their families[10, 13, 14]. Asthma exacerbations are triggered by several environmental factors including allergens, air pollutants[137] and respiratory viral infections, rhinoviruses being the most frequent[138-140]. The mechanisms of viral induced asthma exacerbations are different from those with allergen exposure, possibly explaining the degree of refractoriness to inhaled or oral corticosteroids[141, 142]. The possible up-regulation of epithelia T_H2-type immunity with preferential activation of the immunological cascade as a likely mechanism for the role of epidermal permeability on asthma medication needs has been discussed previously[23]. Similar mechanisms could explain the associations between filaggrin gene defects and the increased risk of asthma exacerbations in children and young adults reported here. However, asthma medication requirements do not necessarily reflect the risk of asthma exacerbations (Figure 4.3.3), while other forms of genetic variation that affect asthma exacerbation risk do not influence asthma medication requirements[24] . Other mechanisms could contribute to the overall picture. Therefore, keratinocytes differentiated in the presence of IL-4 and IL-13 exhibit significantly reduced filaggrin gene expression, suggesting a regulatory role for the atopic immune response on the skin barrier defect[143]. It is possible that multiple mechanisms, including possible interactions between IL-13[30] and filaggrin gene polymorphic variations, could be involved in mediating the observed associations between filaggrin gene defects and the overall burden of asthma (i.e., susceptibility[22], medication requirements[23] and risk of exacerbations[103]).

Chapter 4

Epithelial Permeability Is Increased In Children With Filaggrin-Related Barrier Defects In Skin But Not In Gut

I have observed that the permeability of the skin, as measured by transepidermal water loss, is around 50% higher in children heterozygous for filaggrin gene defects compared to clinically unaffected children but the permeability of the intestinal barrier, as measured by dual sugar absorption tests, is not different. Moreover, while it was confirmed that filaggrin is expressed in human skin[22], no evidence for expression was found in the nasal (inferior turbinate) mucosa or in the oesophagus (proximal, mid or distal) [100].

A recent systematic review and meta-analysis has assessed the overall evidence to demonstrate that filaggrin gene defects increase the risk of developing allergic sensitisation, atopic eczema, and allergic rhinitis [144, 145]. This aspect of my work aimed to explore the biological mechanisms underlying these observations. My work shows that skin but not gut permeability is impaired in children carrying a common genetic defect in the skin barrier protein, filaggrin.

Alterations in gut epithelial permeability have been linked to immunologically mediated disease in children. IgE deposits have been demonstrated on cutaneous Langerhans and mast cells which may account for the cutaneous, intestinal and pulmonary signs observed in Henoch-Schönlein purpura[146]. It is postulated that stimulation of IgE-sensitized mast cells

by specific antigens in the presence of IgA circulating immune complexes may result in the release of vasoactive substances, increased capillary permeability and perivascular deposition of IgA[146]. In addition, the effects of probiotics have been attributed to normalisation of the increased intestinal permeability and balancing gut microecology, improvement of the immunological defence barrier (IgA) of the intestine, alleviation of the intestinal inflammatory response, and downregulation of proinflammatory cytokines characteristic of local and systemic allergic inflammation[147].A similar mechanism might be responsible in development of the atopic diseases. There is need for an exploration of epithelial permeability in gut alongside skin, and the specific role of filaggrin, in various allergies in children, e.g. peanut allergy, cow's milk allergy, egg allergy and house dust mite allergy, to identify the routes of allergen entry (skin, respiratory tract or gastro-intestinal tract), for atopic diseases like asthma and eczema in children.

I have presented evidence that is consistent with a link between filaggrin gene defects and skin permeability. Since filaggrin increases the risk of eczema and asthma, it is inevitable that the disease profile of the filaggrin heterozygous children will be different from children who are representative of the general population. These differences could have secondary effects on skin permeability. For example, skin permeability may be increased in children with asthma and eczema as a result of latent skin inflammation irrespective of filaggrin gene status. Therefore, a much larger study, to define the effects of co-existing, potentially confounding morbidities such as eczema or asthma and the role of other genetic variations, may help establish a causal relationship between filaggrin gene defects, skin permeability and common allergen sensitisation. Such studies, although difficult to perform, will help

improve our understanding of the mechanisms underlying filaggrin-related allergen sensitisation, thus helping the development of intervention trials that aim to reduce the burden of filaggrin-related allergic diseases in children.

Chapter 5:

Future Work

- **Designing a genotype-stratified randomised controlled trial at asthma management step 3 of the British Thoracic society guideline**

An important step in further testing the hypothesis that the study of genotypic variation could contribute to impaired management of children with asthma is through the design of implementation of prospective randomised controlled trials. I have worked with colleagues to design a randomised controlled trial to compare the efficacy of montelukast versus salmeterol as add-on to inhaled fluticasone in at-risk children with asthma possessing the homozygous arginine-16 β_2-adrenoceptor genotype.

In children with asthma managed on step 3 of BTS guidelines for asthma treatment or equivalent, salmeterol provides better asthma control than montelukast[18]. However, in practice, the efficacy of salmeterol for improving asthma control in individual children is often variable[104]. Recently, the US Food and Drug Administration (FDA) have raised concerns regarding the efficacy and safety of long-term salmeterol use in children and adults with asthma[148-150].

We have identified of a step-wise increase in the risk of asthma attacks per copy of the Arg16 allele on the *ADRB2*, in asthmatic children on regular salmeterol[21, 104]. This led to

the hypothesis that children carrying this allele could be less responsive to salmeterol and may experience relatively better asthma control with montelukast. My observational data indicated that any such difference in efficacy could be more prominent in children who are homozygous, rather than heterozygous, for the Arg16 allele, as they carry the maximum 'gene dose'[104]. If a significant difference in efficacy exists in practice, prior testing for the Arg16 allele may predict response and guide choice of 'controller' therapy, thereby reducing the risk of treatment failure or deterioration with salmeterol in a proportion of these children, while improving its safety profile. As 1 in 7 in the population is homozygous for this allele[64, 117], such predictability of response may improve the cost-effectiveness of asthma therapy in children.

The addition of anti-leukotrienes, or the substitution of long-acting inhaled β_2-agonists with other medication, could alter the response. Receptor desensitization and down-regulation has been described with the chronic use of β agonists. This effect may not be the same with all beta-agonists and may be related to their stabilization of altered receptor states.

As a follow-up to the research reported above, I am working with my colleagues to perform a proof-of-principle randomised controlled trial to test the hypothesis that genetically susceptible children who possess the homozygous for the Arg16 allele might experience superior long-term asthma control with montelukast compared to salmeterol as add on to inhaled fluticasone. We have designed the study as follows.

The BREATHE database is used to identify children (5-18 years) homozygous for the Arg16 genotype variation for participation in this study. 62 children currently on regular

inhaled corticosteroids as preventer medication for asthma have been recruited. All participants had a history of at least one of the following within the previous 12 months as a result of asthma: school absences, course of oral steroids, out-of-hours visits to general practice or hospital admissions.

Participants are randomly allocated to one of two treatments at the screening visit- Flixotide® (fluticasone propionate) via accuhaler (Diskus) dry powder inhaler device as per current inhaled steroid dose plus oral montelukast; or Seretide® (salmeterol plus equivalent dose of fluticasone) via accuhaler dry powder inhaler device as per current inhaled steroid dose plus placebo for montelukast. The study is designed as a pragmatic randomised controlled trial. The trial is not blinded as the inhalers are not packaged in identical dry powder inhalers.

Participants are randomly assigned to the two groups at baseline visit, by using concealed web-based randomisation process. At baseline visit, all participants undergo detailed clinical examination. Exhaled nitric oxide (Aerocrine, Solna, Sweden)[151] and pulmonary function (forced expiratory volume at 1 second (FEV_1), forced vital capacity (FVC), peak expiratory flow rate (PEFR), forced expiratory flow between 25% and 75% of vital capacity ($FEF_{25\%-75\%}$)) (Micromed, Rochester, United Kingdom) are measured at baseline. Patients are provided with an asthma symptom diary to record controller and reliever medication use and exacerbation symptoms. Participants return every 3 months for diary review, medication compliance review, spirometry, exhaled nitric oxide testing and safety and efficacy assessments. A standard protocol is followed for spirometry[106]. Compliance is

monitored by viewing counters that calculated the number of actuations used from the accuhaler. The diary cards are used as a secondary compliance check.

School absence, prospectively measured as individual events over 1 year, constitutes the primary outcome measure. Secondary outcome measures include asthma-related hospitalisations, requirement of courses of oral steroids, daily asthma symptoms as reported by the participants, quality of life as measured by the Juniper paediatric asthma quality of life questionnaire (PAQLQ) [152,153], nitric oxide levels and spirometry.

- **A Proof-of-Concept Study to evaluate the benefit from ipratropium bromide versus salbutamol as reliever in children with asthma carrying the Arg16 variation of β_2-receptor genotype**

My work has shown that there is an increased risk of asthma attacks in a particular genetic configuration (Arg16 heterozygous and homozygous status on the β_2-receptor gene) in children and young adults with asthma on frequent (once daily or more) as required doses of inhaled salbutamol, a common reliever medicine acting via β_2-receptor[104] . This effect was not observed in participants with asthma who are not exposed to inhaled β_2-agonist on a daily basis. The interaction between genotype and daily use of any inhaled β_2-agonists (as required salbutamol and/or salmeterol) had a significant effect on the risk of asthma exacerbations (p=0.022)[104]. As the participants on inhaled long-acting β_2-agonists were also on short-acting β_2-agonists, what proportion of the adverse effect of increased risk of exacerbations in the 'real-life' scenario results through the use of long-acting, in comparison to the use of short-acting, β_2-agonists could not be explored. However, this question may be resolved through a study that involves the randomization of asthmatic children carrying the Arg16 allele to comparator arms of 'reliever' medication, short-acting β_2-agonist versus ipratropium bromide (an anticholinergic agent which decreases bronchial smooth muscle contractility and does not act via the β_2 receptor), with risk of asthma exacerbations as the primary outcome measure.

There was an increase in the risk of school absences and requirement for courses of oral steroids due to exacerbations over a 6-month period of reporting, in addition to the overall

effect on asthma exacerbations[104]. However, there was no significant effect of genotype and short-acting β_2-agonist use on the risk of hospital admissions. School absences, requirement of oral steroids and hospital admissions due to asthma, represent different aspects of asthma exacerbations that have different effects on children and young adults in the community. School absences have an effect on children's education, and oral steroid courses represent the use of primary care resources, whereas hospital admissions represent the effect on secondary care services. Therefore my findings appear to have a greater relevance for the burden of asthma in the community and primary care than hospital settings.

My primary hypothesis for the proposed study is that children with asthma, carrying the Arg16 variation of the β_2-receptor genotype experience better asthma control including a lower risk of exacerbations over 1 year if maintained on reliever therapy with inhaled ipratropium bromide compared to salbutamol. The secondary hypothesis is that there is an improvement in asthma specific quality-of-life in children with asthma, carrying the Arg16 variation of the β_2-receptor genotype on inhaled ipratropium in comparison to inhaled salbutamol over a period of 1 year.

I would like to perform a randomized controlled trial (parallel design) of inhaled ipratropium versus inhaled salbutamol as reliever on asthmatic children treated with inhaled steroids, with a history of any measure for exacerbations over the past year, using asthma exacerbations over a period of 1 year as the primary outcome.

Children with and without the Arg16 allele will be recruited from the genotyped population of asthmatic children. The primary hypothesis to be tested is that inhaled ipratropium is associated with reduced school absence in comparison to inhaled salbutamol during exacerbation over a period of 1 year in asthmatic children with Arg16 allele.

The secondary outcome measures for the proposed study is that inhaled ipratropium is associated with reduced overall asthma exacerbations and improved asthma specific quality-of-life during exacerbation in comparison to inhaled salbutamol over a period of one year.

This study is important in the context of personalization of treatments according to genotype, which may represent a new therapeutic strategy for children with a common disease. My work intends to help target reliever therapy to make treatment more effective, thereby reducing the burden of childhood asthma. School absences have an effect on children's education, and oral steroid courses represent the use of primary care resources. Therefore this study will have a greater relevance for the burden of the disease in the community and primary care.

SECTION VII

CONCLUSION

I have explored the associations of two common genotypes with increased risk of exacerbations in children with asthma. I have also shown that the most commonly used broncho-dilator may in the long term worsen asthma exacerbations in a susceptible group of children with asthma. Further exploration of the pathway(s) via which these gene variations affect the disease outcome may lead to a clearer understanding of these effects and help the introduction of genotype-specific treatment of a common chronic disease. Reduction in the disease burden of childhood asthma can therefore be achieved by targeting the therapy according to the genotype variations.

The identification of a gene and subsequently its disease associations may eventually lead to genotype-directed treatment. This process includes replication of earlier findings, functional studies to identify mechanisms of the gene action and, finally, intervention studies. Significant progress has been made in the identification of asthma susceptibility genes. Further research is required for replication and characterization of the gene function. This may enable us to realize benefits to patient treatment that studies of the genetic basis of asthma have the potential to deliver.

- **Further exploration of the gene mechanisms influencing disease outcomes**

β_2-adrenergic receptors are activated by β_2-agonists such as salbutamol or salmeterol. This leads to the activation of the cascade of events by targeting β_2-adrenergic receptors. Signalling through β_2-adrenergic receptors mediates direct relaxation of airway smooth muscle and consequent broncho-dilation pathways. Stimulation of β_2-adrenergic receptors on inflammatory cells like mast cells, eosinophils, neutrophils and lymphocytes, activates a signalling cascade that inhibits the release of inflammatory mediators and cytokines[154]. Therefore, it is an important prerequisite to understand these pathways, which will assist in defining the variability in therapeutic responses for β_2-agonists[155]. Owing to the complex therapeutic response of the β_2-agonists, a broader genomics approach will help in targeting asthma therapy for the susceptible patients. This could be achieved by focusing either on the receptors at which the drug binds directly, signal transduction cascades, or downstream proteins and proteins involved in the relaxation of the airway smooth muscle.

Filaggrin protein has not been isolated from the respiratory tract[100]. Yet asthmatic children with *FLG* null mutations are at increased risk of asthma exacerbations[105]. Therefore functional studies are essential to define the underlying mechanism for the association of asthma exacerbations with *FLG* null mutations. Replication studies of the previously reported association of the FLG mutations R501X and 2282del4 with atopic eczema and eczema-associated asthma show that these mutations predispose equally to the atopic and nonatopic forms of eczema, suggesting that the onset of eczema may occur independently

of allergic sensitization. It has also been demonstrated in a population-based cohort that the defect in a single gene may predispose to different allergic phenotypes of the atopic march and that the expression of the subsequent disease stages requires the presence of eczema[156]. These findings highlight the role of the epidermal barrier in the pathogenesis of allergic sensitization and allergic disease and also stress the importance of maintaining or repairing the epidermal barrier in infantile eczema as a possible means of preventing allergic airways disease.

- **How to reduce the disease burden of childhood asthma**

Exacerbations not only affect patients' quality of life, it also account for the largest proportions of health costs of asthma[136]. 43% of children reported to have been prevented from doing everyday activities due to their asthma[157]. The estimated cost of treating a child with asthma (£181) is higher than the cost per adult with asthma (£162)[157]. Asthma costs the UK approximately £2,237 million per year[16], and the US approximately $37.2 billion per year[158]. In the UK lost productivity accounts for the direct costs to the NHS of around £889 million and £161 million in benefits[159]. Difficult to control asthma costs the NHS £680 million a year[15] and emergency hospital admissions for asthma cost the NHS more than £45.8 million a year[15]. Asthma care costs a primary care trust over £4.25 million a year[160].

Even a relatively small reduction in the emergency visits to general physicians through such genotype-targeted reliever therapy might not only be cost-effective, but also

significantly improve the quality of life of the children. However, introducing pharmacogenetics into the clinical practice can be quite complex. There is always a context to a pharmacogenetic test when it enters a clinical situation[161]. The contextual approach starts from the clinical experience, looking at the ethical aspects of pharmacogenetics as seen by the people beginning to use this new technology. There is a substantial role of clinicians' 'resistance' to various aspects of pharmacogenetics[161], with resistance being a political term referring to 'elements of discourse that seem to conflict with or evade more dominant assumptions and priorities'[162].

Prescribing policies get set by healthcare providers and funders, and if there were definitive tests that would predict response on a genetic basis, there might be a pressure from the health authority and government level to focus prescribing[161]. Such decisions, made at the population level need not necessarily be in harmony with the wishes and needs of the individual, or people closely related to that individual[161]. In this sense, the needs of the individual patient are in conflict with those of society at large.

- **Exploring the context of a genotype-directed management in current clinical practice: The example of Herceptin**

Herceptin, the pharmacogenetic product currently being prescribed by clinicians for breast cancer in patients with HER2 gene variations, is in many ways subject to bureaucratic pressures[161]. These go beyond the issues involved in any normal expensive treatment; the

point about pharmacogenetics being that a test will be required for the drug to be prescribed properly. Yet there are several levels at which Herceptin is controlled and limited, largely on economic grounds. Herceptin was developed with the intention of being prescribed to a genetically identified sub-group of patients. As the first pharmacogenetic drug licensed in the US (in 1998) and the UK (a year later), Herceptin is often cited as a pharmacogenetic success story, although such a claim rather glosses over the number of times the drug was almost cancelled during development[163].

There may be important ethical problems with pharmacogenetics, but they may be overridden by the benefits like potential gains from pharmacogenomics, in terms of both patient well-being and cost of healthcare, and heavily outweigh the risks[164]. Requirements for confidentiality and limitation of using the genetic data place major constraints on the ability to use the genetic information in modifying the disease outcomes. Therefore the need for education, information, and teaching about genetics is large and evident, to put risks in perspective and illuminate the potential benefits[165]. One obvious ethical result of the benefits brought by pharmacogenetics is that one day 'it may be considered unethical not to carry out such tests routinely to avoid exposing individuals to doses of drugs that could be harmful to them'[166]. Hence testing the genotype of asthmatic children to avoid exposure to $\beta_2$agonist in susceptible population will be an important step forward in treating childhood asthma.

- **Pharmacogenetics in childhood asthma**

The new US and EU regulations on medicines emphasize the need for the pharmacogenetic approach[167]. Although the pathophysiology of childhood and adult asthma may share the same underlying mechanisms, effects on growth and development, and the adverse effects of asthma treatments differ between children and adults [129,168]. There is a need for successful paediatric clinical trials safeguarding the participants as well as focusing on the outcome measures relevant to the paediatric population[169-171].

While there is considerable literature related to the genetic testing of children, both for childhood and later onset adult conditions[172], there has been very little discussion of the ethics of pharmacogenetic research on children. What work there has been in this area highlights the complex issues around informed consent to such trials (from both children and their parents), the limitations on risks that can be taken with children, and the complicated genetics involved in those cases where genes are expressed differently in children compared to adults[173].

One obvious clarification concerns the nature of the gene being tested for, and whether there is any 'overlap' between a gene's role in the pharmacogenetics of a particular drug, and disease prediction or prognosis. This 'ancillary information' in pharmacogenetic testing may be more common than is often assumed[174] and there are some examples where the ethically contentious nature of this additional information

has inhibited the clinical uptake of pharmacogenetic tests[175].

- **Ethical issues**

Pharmacogenomic approach is not without its challenges, not the least of which is the ethics questions surrounding the privacy of genetics data[176]. It may not necessarily be the case that a clear line can be drawn between a pharmacogenetic test and a test for disease susceptibility as genotypes relevant to drug response may overlap with disease susceptibility, and divulging such information could jeopardise an individual[177]. Genotypes related to drug metabolism or mechanisms are often related to the diseases[178].

Although the ethical debates around pharmacogenetics are largely being set by scientific and commercial considerations[179], the bioethicists have also explored potential problems that may arise with pharmacogenetics[180-182]. The emphasis on the ethical issues involved in pharmacogenetic research, debates surrounding informed consent. In the case of pharmacogenetics, the worries are that patients may not understand the reason their DNA is being sampled, or that the imprecise nature of much pharmacogenetic research means that, by definition, it is not possible to give patients full information about the uses to which their DNA may be put. Related to this is the concern that their genetic data may 'leak' into the broader community due to inadequate confidentiality and may thus lead to social or financial stigmatisation[183]. Although it is ensured that the patients' DNA, personal, and medical data are encoded to prevent any infringement of privacy, 'full anonymisation' (i.e. the removal of all identifiers, making tracing the patient all but impossible) might prevent

us feeding back results to patients. There are also the potential problems which may arise from the clinical application of pharmacogenetics. Although there are major personal implications of clinical pharmacogenetics, such as the impact on insurance[184, 185], there could be broader, systemic issues that will arise, such as physician (and pharmacist) education, the direct marketing of pharmacogenetic tests to the public, and possible issues of legal liability[186-188].

Therefore obtaining informed consent within the context of pharmacogenetic testing and its application in management of childhood asthma is of immense importance. Respecting patients' autonomy in making decisions over treatment, and getting their 'informed consent', is at the core of modern medical ethics. Both in terms of the rules laid down for ethical clinical practice[189, 190] and in philosophically based medical ethics[191], the need to get informed consent from patients is crucial. Yet a considerable amount of empirical research suggests just how hard it is to decide exactly what counts as 'informed' consent[192]. Prior implementation of the pharmacogenetic testing in routine practice to treat such a common childhood disease, there is a need to conduct genotype-specified clinical trials in children to answer specific questions of clinical importance.

- **Parental views on pharmacogenetics and genotype-specific clinical trials in children**

In general, parents felt motivated to consent their children entering pharmacogenetic clinical trials because there was a possible perceived benefit for their own child found

to carry the genotype, but also the satisfaction of knowing they may be helping other children with asthma in the future. The participants and their parents were interested in contributing to the understanding of the gene-environment interactions in childhood asthma and allergy. Most parents welcomed being contacted with follow-up genetic information leading to further studies that could help the management of specific issues that concerned their child.

The current devolution of budgets within healthcare providers can cause problems not in funding for the drug, but the funding for the genetic testing, which is not routinely done. It is of course ironic that in this case there is enough money for the relatively expensive treatment, but no budget for the test. Using various measurement tools for quantifying risk, various forms of genetic variation, such as for the β_2 adrenergic receptor gene, have been shown to influence the likelihood of asthma exacerbations, possibly through an interaction with pharmacological treatments[21,29,30]. I predict that the study of genetic variation in relation to clinical outcomes in asthma will further explain underlying mechanisms for this disease, identify at-risk populations for susceptibility, severity and major life-events, define drug choice, and contribute overall to significantly improved management strategies for asthma in the future and will also have a major implication in cost-reduction for the National Health Service. I hope this also provides a platform for further discussion on the methodology of prospective genotype-stratified trials in children; as such trials are likely to become more relevant to the progress in pharmacogenetic therapeutics in the future.

SECTION VIII

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APPENDIX

- **Publications from the work presented in the thesis**

1. **Basu K**, Palmer CNA, Tavendale R, Lipworth BJ, Mukhopadhyay S. Adrenergic β2-receptor genotype predisposes to exacerbations in asthmatics on as required albuterol and regular salmeterol. J Allergy Clin Immunol.*(Impact factor 9.773)* 2009 Dec;124(6):1188-94.e3.
2. **Basu K,** Palmer CNA, Lipworth BJ, McLean WHI, Terron-Kwiatkowski A, Zhao Y, Liao H, Smith FJD, Mitra A, Mukhopadhyay S. Filaggrin null mutations are associated with increased asthma exacerbations in children and young adults. Allergy. *(Impact factor 6.204)* 2008 Sep; 63(9):1211-7.

- **Abstracts from the work presented in the thesis**

1. **Basu K**, Palmer CNA, Tavendale R, Lipworth BJ, Mukhopadhyay S. Adrenergic β_2-receptor genotype predisposes to exacerbations in asthmatics on as required short acting and regular long acting β_2 agonists. Oral presentation for the award of Runner up- Young Investigator at the British Thoracic Society winter meeting 2008
2. **Basu K**, Palmer CNA, Lipworth BJ, McLean WHI, Terron-Kwiatkowski A, Zhao Y, Liao H, Smith FJD, Mitra A, Mukhopadhyay S. Filaggrin Null Mutations May Predict Increased Risk Of Asthma Exacerbations In Children And Young Adults.

Oral presentation at the Royal College of Paediatrics and Child Health annual spring meeting 2008

3. **Basu K.** The effect of gene variations on exacerbations and treatment preferences in children's asthma- Poster presented at the annual postgraduate symposium, University of Dundee- July 2008
4. **Basu K.** The effect of β_2 agonist receptor gene variation on treatment preferences in children's asthma- Oral presentation at the annual postgraduate symposium, University of Dundee- June 2007